Early Steps in Cotranslational Translocation of Proteins across the ER Membrane: A Biochemical and Structural Analysis

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ZUSAMMENFASSUNG

Sekretorische Proteine und Proteine der Kompartimente des sekretorischen Transportweges müssen die Membran des Endoplasmatischen Retikulums überqueren, um an ihren Wirkungsort zu gelangen. In der vorliegenden Arbeit wurden frühe Schritte des kotranslationalen Transports von Proteinen durch die ER-Membran untersucht. Signalsequenzen leiten diese Proteine als ribosomengebundene Intermediate an die ER-Membran. Die Ribosomen binden dort an den Sec61p-Komplex, der als Ribosomenrezeptor wirkt und gleichzeitig den proteinleitenden Kanal in der Membran bildet. Die Assoziation von Ribosomen mit dem Sec61p-Komplex verläuft in zwei Phasen. Die initiale Bindung ist sensitiv gegenüber hohen Salzkonzentrationen. Die Ribosomenbindung wird salzresistent, wenn die naszierende Kette in den Kanal inseriert und der Sec61p-Komplex die Signalsequenz erkennt. Sowohl Ribosomen ohne naszierende Kette als auch Ribosomen, die Proteine ohne Signalsequenzen synthetisieren, sind nur zur initialen salz-sensitiven Bindung an den Sec61p-Komplex fähig.

Signalsequenzen interagieren im Cytosol mit SRP (engl.: Signal Recognition Particle). In dieser Arbeit wurde gezeigt, daß Signalsequenzen außerdem von Calmodulin gebunden werden. SRP und Calmodulin scheinen für die Interaktion mit Signalsequenzen einen ähnlichen Mechanismus zu benutzen, der wiederum mit der Signalsequenzerkennung durch den Sec61p-Komplex verwandt ist.

Alle Ribosomen, unabhängig davon ob und welches Protein sie translatieren, können mit dem Sec61p-Komplex interagieren und daher um Bindungsplätze an der ER-Membran kompetitieren. Wenn SRP an die Signalsequenz einer naszierenden Kette gebunden ist, erhalten diese Ribosomen jedoch einen Vorteil in der Kompetition. Nur sie können Ribosomen ohne naszierende Kette oder Ribosomen, die ein cytosolisches Protein translatieren, vom Sec61p-Komplex verdrängen und sich selbst dann einen Translokationsort sichern, wenn alle Bindingsplätze an der Membran besetzt sind.

In der vorliegenden Arbeit wurden dreidimensionale Strukturen von Komplexen aus Ribosom und proteinleitendem Translokationskanal vorgestellt, die der ersten und und zweiten Phase der Ribosomenbindung entsprechen. Überraschenderweise unterscheiden sich diese beiden Stadien strukturell nicht. In beiden Fällen existieren definierte Verbindungen zwischen Ribosom und Kanal, die eine Lücke von etwa 20Å zwischen dem Ribosom und der Membranoberfläche überbrücken. Die Lücke stellt eine Verbindung zum Cytosol her, die eventuell dazu dient, naszierende Ketten ins Cytosol zu entlassen, wenn diese nicht ins Lumen des ER transportiert werden sollen. Weiterhin zeigen wir, daß der Kanal in nativen Membranen größer ist als der Kanal, der nur aus gereinigtem Sec61p-Komplex besteht. Dieser größere Kanal besitzt eine zusätzliche lumenale Domäne, die von der Oligosaccharyltransferase oder vom TRAP-Komplex gebildet wird.

Schlagworte: Endoplasmatisches Retikulum, Proteintransport, SRP, Ribosom, Sec61p-Komplex

SUMMARY

The first step in the secretory pathway is the translocation of proteins across the membrane of the endoplasmic reticulum (ER). In this thesis project, early stages of cotranslational protein translocation in mammalian cells were studied.
Proteins following the secretory pathway are targeted to the ER as ribosome-nascent chain complexes by their N-terminal hydrophobic signal sequences. The nascent chain is translocated across the ER membrane through a hydrophilic channel formed by the Sec61p complex, which also functions as the ribosome receptor. The initial binding of ribosomes to the ER membrane is salt-sensitive. After insertion of the nascent chain into the translocation channel and signal sequence recognition by the Sec61p complex, the ribosome is bound in a salt-resistant manner. The membrane binding of ribosomes lacking nascent chains and of ribosomes carrying nascent chains without signal sequences is always salt-sensitive.

It is known that in the cytosol, the signal sequence binds to the signal recognition particle (SRP). Here we show that another cytosolic factor, the small regulatory protein calmodulin, can interact with signal sequences. Our data suggest that both SRP and calmodulin use a similar mechanism for substrate binding and recognition. In fact, this mechanism may be related to signal sequence recognition by the Sec61p complex.

Previously the question has been raised of how efficient targeting of ribosome-nascent chain complexes (RNCs) carrying a signal sequence is possible when all ribosomes, regardless of the presence or nature of a nascent chain, can bind to the Sec61p complex. We demonstrate that all ribosomes compete for common binding sites at the ER membrane and that SRP functions as a positive effector to give RNCs carrying a signal sequence an advantage over other ribosomes. RNCs with a signal sequence and bound SRP can displace ribosomes without a nascent chain and ribosomes synthesizing cytosolic proteins from the membrane and can therefore secure a translocation site even when all ribosome binding sites at the ER membrane are occupied.

A structural analysis by single particle cryo electron microscopy revealed that ribosome-translocation channel complexes do not differ in the salt-sensitive or the salt-resistant stage of ribosome binding to the ER membrane. Furthermore our data show that the ribosome is linked to the translocation channel by a discrete number of connections. Even in the presence of a translocating nascent chain the ribosome-membrane junction is not completely sealed towards the cytosol. Instead, a sizable gap exists between the ribosome and the surface of the membrane that may allow nascent polypeptide chains to enter the cytosol when their translocation across the ER membrane is prevented. We also show that translocation channels derived from native microsomes are larger than channels derived from purified Sec61p complex. These larger channels contain a wider central pore and an additional lumenal domain, which is formed by the oligosaccharyl transferase or by the TRAP complex.

Key words: Endoplasmic reticulum, Protein translocation, SRP, Ribosome, Sec61p complex

CONTENTS

1. INTRODUCTION

Eukaryotic cells contain membrane-enclosed compartments, such as mitochondria, nuclei and lysosomes. Each of these organelles is equipped with a unique set of proteins to fulfill specific tasks within the cell. Therefore, sorting of proteins to their correct location is a critical step for establishing and maintaining the identity of organelles.

Proteins destined to be secreted from the cell or for residence in the plasma membrane, the lysosomes, the Golgi apparatus or the endoplasmic reticulum (ER) follow the secretory pathway to their destination. The first step of the secretory pathway is targeting of the nascent protein to the membrane of the endoplasmic reticulum and subsequent translocation across the membrane into the ER lumen or, in case of membrane proteins, the integration into the phospholipid bilayer (Palade, 1975). During later stages of the secretory pathway transport vesicles shuttle proteins from the ER to the plasma membrane and lysosomes via the Golgi apparatus.

Protein translocation across the ER membrane (for review see Rapoport et al., 1996a) can occur while the protein is being synthesized by the ribosome (cotranslational translocation) or after translation has been completed (posttranslational translocation). In mammalian cells most proteins are translocated cotranslationally. In the yeast Saccharomyces cerevisiae, however, both the co- and the posttranslational pathway are used.

1.1. The Sorting Signal for ER Translocation

The signal for ER-targeting is an N-terminal hydrophobic peptide of approximately 15-30 residues (Blobel and Dobberstein, 1975; for review see Gierasch, 1989; Martoglio and Dobberstein, 1998). The primary structure of these signal sequences is not conserved, instead they share characteristic features (von Heijne, 1985). A hydrophobic core of about 8-15 residues is preceded by a short polar region (N-domain) which may contain charged amino acids.

The region following the hydrophobic stretch generally contains small polar residues. The hydrophobic core is thought to form an α-helix (von Heijne, 1985). It has also been shown that isolated signal peptides can adopt an α-helical conformation in nonpolar environments (Gierasch, 1989; McKnight et al., 1989).

1.2. Cotranslational Targeting of Nascent Polypeptides to the ER Membrane

In cotranslational translocation, targeting to the membrane of the endoplasmic reticulum begins when the signal sequence of the nascent polypeptide chain emerges from the ribosome (for review see Walter and Johnson, 1994). A cytosolic factor, the signal recognition particle (SRP) has been shown to bind to signal sequences (Walter et al., 1981). SRP consists of the 7SL RNA and six polypeptides (Walter and Blobel, 1980; 1982). The 54kDa subunit of SRP (SRP54) interacts with signal sequences (Krieg et al., 1986; Kurzchalia et al., 1986). SRP54 comprises a GTPase domain (Bernstein et al., 1989; Römisch et al, 1989) and a methionine-rich M-domain that has been shown to contain the signal sequence binding site (Zopf et al., 1990; High and Dobberstein, 1991; Lütcke et al., 1992). It is thought that the highly flexible side chains of methionines line the walls of a hydrophobic pocket, forming a "methionine- bristle" that would allow recognition of a wide variety of signal peptides (Bernstein et al., 1989). A structural analysis of a bacterial homologue of SRP54 confirmed the existence of a hydrophobic groove in the M-domain (Keenan et al., 1998).

Ribosomes carrying a nascent polypeptide with bound SRP are targeted to the endoplasmic reticulum by two affinities: The ribosome interacts with its membrane receptor, the Sec61p complex (Görlich et al., 1992a; Kalies et al., 1994; Jungnickel and Rapoport, 1995), and SRP interacts with the SRP receptor (SR; Gilmore et al., 1982a; 1982b; Meyer et al., 1982). After binding of SRP to SR, the signal sequence is released from SRP54 (Connolly and Gilmore, 1989; Rapiejko and Gilmore, 1997). It is now free to contact the translocation site at the ER membrane (translocon) and subsequently protein translocation is initiated.
The SRP receptor has two subunits, SRα and SRβ. Like SRP54, they both contain GTPase domains (Connolly and Gilmore, 1989; Miller et al., 1995). In fact, an analysis

of the crystal structure of Ffh, the bacterial homologues of SRP54, and of FtsY, the homologue of SRα, suggests that their GTPase domains are related and constitute their own subfamily of GTPases (Freymann et al., 1997; Montoya et al., 1997).

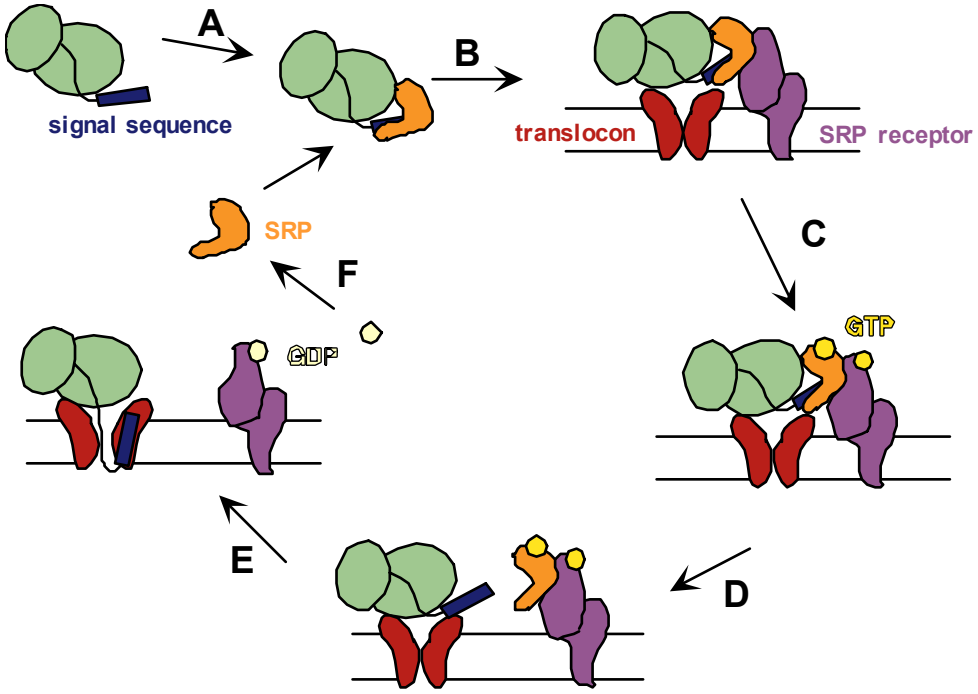

Figure 1: SRP-mediated targeting. A: When a signal sequence emerges from the ribsosome, it is bound by the nucleotide-free form of the 54kDa subunit of signal recognition particle (SRP54). B: The ribosome-nascent chain complex with bound SRP is then targeted to the ER membrane by two interactions: SRP binds to the SRP receptor (SR) and the ribosome contacts the Sec61p complex. C: The affinity of SRP54 for GTP is increased upon binding to SR. D: GTP binding to SRP54 results in the release of the signal sequence which can now interact with the α subunit of the Sec61p complex. Subsequently, translocation begins. SRP-GTP remains bound to SR. In the GTP-bound state, SRP54 and SRα reciprocally stimulate their GTPase activity. E: After GTP hydrolysis, SRP dissociates from SR and releases GDP. F: SRP54 remains in a nucleotide-free form until SRP engages in the next round of targeting.

The GTP cycles of SRP54 and SRα regulate targeting of RNCs to the ER membrane (Figure 1; see Rapiejko and Gilmore, 1997): According to a current model nucleotide-free SRP54 binds to a signal sequence (Rapiejko and Gilmore, 1997). The complex is thought to remain in a state of low affinity for GTP. Interaction of SRP with the ribosome might increase the affinity of SRP for GTP (Bacher et al., 1996). A further increase occurs upon targeting of the RNC-SRP to the ER membrane and interaction of SRP with its receptor (Miller et al., 1993; Rapiejko and Gilmore, 1997). It has been proposed that GTP-binding to SRP54 causes a conformational change that results in the release of the signal sequence, allowing translocation to begin (Connolly and Gilmore, 1989). SRP-GTP remains associated with SR and would dissociate only after GTP hydrolysis (Connolly et al., 1991). It has been shown that SRP54 and SRα act as

GTPase-activating proteins for each other while they are in their GTP-bound form (Powers and Walter, 1995). After GTP hydrolysis SRP54 releases GDP and remains in a nucleotide-free form until the next round of targeting (Connolly et al., 1991; Rapiejko and Gilmore, 1997). The release of GDP without a nucleotide exchange factor and the stable nucleotide-free stage of SRP54 (Freymann et al., 1997; Freymann et al., 1999) are an unusual feature of this GTPase.

1.3. The Sec61p Complex

Both the release of the signal sequence from SRP54 and the binding of the translating ribosome to the ER membrane are prerequisites for the nascent chain to engage in translocation. But how does a hydrophilic polypeptide cross a hydrophobic membrane? A protein-conducting channel in the membrane of the endoplasmic reticulum had been proposed (Blobel and Dobberstein, 1975; Gilmore and Blobel, 1985) and electrophysiology studies have provided evidence for its existence (Simon and Blobel, 1991). Furthermore, fluorescence quenching data suggested that the nascent chain is in an aqueous environment while it is crossing the membrane (Crowley et al., 1993). The heterotrimeric Sec61p complex has been identified as a central component of the translocation machinery. In mammals, the Sec61p complex contains an α subunit with 10 membrane-spanning domains, and β and γ subunits, each of which spans the membrane once (Figure 2; Hartmann et al., 1994). Initially, crosslinking experiments provided evidence that the Sec61p complex forms the actual channel (Mothes et al., 1994; for review see Martoglio and Dobberstein, 1996). The Sec61p complex has been found to be tightly associated with membrane-bound ribosomes and it also functions as the ribosome receptor (Görlich et al., 1992a; Kalies et al., 1994). Using a photocrosslinking approach it has been shown that the α subunit of the Sec61p complex is in proximity to nascent polypeptide chains throughout their transfer across the membrane (Mothes et al., 1994).

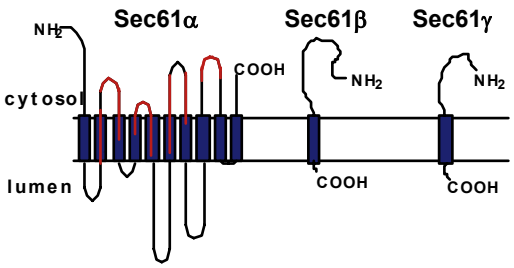

Figure 2: The membrane topology of the mammalian Sec61p complex. Sec61α is an integral membrane protein with 10 membrane spanning domains. It is highly homologous among different species. The conserved regions are shown in red. Sec61β and Sec61γ are tail-anchored membrane proteins with their only membrane spanning domain located at the C-terminus.

Additional evidence that the Sec61p complex forms the translocation channel came from experiments utilizing reconstituted proteoliposomes. Purified membrane proteins and pure lipids can be used to generate proteoliposomes of a defined composition. The Sec61p complex and SRP receptor have been shown to be necessary and sufficient for translocation of the secretory proteins preprolactin, Kar2p and pre-growth hormone (Görlich et al., 1992a; Voigt et al., 1996). Since the function of SR is SRP binding and since SR only contains one membrane-spanning domain, it has been concluded that the Sec61p complex forms the protein conducting channel and that it acts as the ribosome receptor.

The Sec61p complex is evolutionary well conserved from bacteria to man (Hartmann et al., 1994; for review see Matlack et al., 1998). In the budding yeast S. cerevisiae, Sec61p, a homologue of Sec61α, was found in a genetic screen (Deshaies and Schekman, 1987). It forms the Sec61p complex together with Sbh1p and Sss1p, the homologues of mammalian Sec61β and γ. In E. coli, SecY and SecE are homologues of Sec61α and Sec61γ, respectively. Together with SecG, they form the SecYEG complex, the major component of the translocation channel in the bacterial inner membrane (Hanada et al., 1994; for review see Ito, 1995).

1.4. The Sec61p Complex Forms Ring-Like Structures

Further evidence that the Sec61p complex forms the protein conducting channel in the ER membrane comes from electron microscopy studies. The purified Sec61p complex from mammals and S. cerevisiae and the purified SecYEG complex all assemble into ring-like structures in detergent solution (Hanein et al., 1996; Meyer et

al., 1999; Manting et al., 2000). The size of these rings suggest that each contains three or four copies of the Sec61p or SecYEG complex, in good agreement with the finding that in mammals membrane-bound ribosomes are associated with 3 to 4 copies of the Sec61p complex (Hanein et al., 1996).
Channel-like structures have also been detected in native ER membranes examined by freeze-fracture electron microscopy (Hanein et al., 1996; Meyer et al., 1999). Yet, when purified mammalian Sec61p complex was reconstituted into proteoliposomes, no rings were seen. However, the ring-like structures reappeared when the membranes were incubated with ribosomes before electron microscopy. This was confirmed with proteoliposomes containing the yeast Sec61p complex. Again, ring-like structures were seen after addition of ribosomes to the vesicles. Interestingly, the same structures appeared after coreconstitution of the Sec62/63p complex, a tetrameric complex of membrane proteins that interacts with the Sec61p complex to facilitate posttranslational translocation of proteins (Deshaies et al., 1991). This suggests that oligomerization of the Sec61p complex may be induced by the interacting partner(s) in either co- or posttranslational protein transport.

Recently, a structure of yeast nontranslating ribosomes bound to S. cerevisiae Sec61p complex in detergent solution was determined by single particle cryo electron microscopy (Beckmann et al., 1997). This 3D map shows a channel very similar to the ones described above. The ribosome is separated from the Sec61 channel by a sizable gap, bridged only by a single connection. One goal of this thesis was to determine the structure of a ribosome-nascent chain-Sec61p complex to ascertain whether the ribosome-channel junction becomes more intimate when a nascent chain is inserted into the translocation channel.

1.5. Early Stages of Cotranslational Translocation Across the ER Membrane

Ribosome-nascent chain complexes are targeted to the endoplasmic reticulum by the SRP pathway and by the affinity of the ribosome for the Sec61p complex. Using early translocation intermediates two distinct stages of ribosome-membrane interaction have been characterized.

First the ribosome-nascent chain complex (RNC) is bound only loosely to the Sec61p complex (Figure 3A; Jungnickel and Rapoport, 1995). This initial ribosome-Sec61p interaction is salt-sensitive (see also Wolin and Walter, 1993) and the nascent chain is accessible to cytosolic proteases. However, even at this early stage the signal sequence contacts the α subunit of the Sec61p complex, as shown by photocrosslinking experiments. Upon elongation of the nascent chain, at a length of about 70 residues for the secretory protein preprolactin, a transition to tight binding of the RNC to the channel takes place (Figure 3B; Jungnickel and Rapoport, 1995).

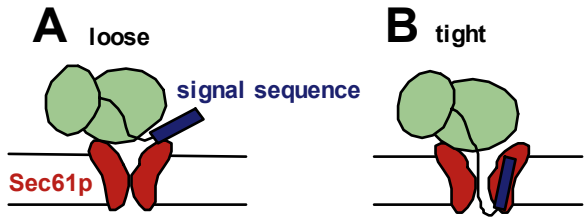

Figure 3: Loose and tight stages of ribosome binding to the Sec61p complex. A: The initial interaction between a ribosome-nascent chain complex carrying a signal sequence and the Sec61p complex is sensitive to high salt concentrations. The nascent chain remains sensitive to protease digestion. B: Upon elongation of the nascent chain, the signal sequence inserts into the translocation channel and is recognized by the Sec61p complex. The interaction between ribosome and Sec61p channel is now resistant to high salt concentration and the nascent chain becomes protected from protease digestion.

The interaction between ribosome and Sec61p complex becomes resistant to high salt concentrations (Wolin and Walter, 1993; Connolly and Gilmore, 1986). Furthermore, the nascent chain is protected against added proteases. Fluorescent probes incorporated into the nascent chain cannot be quenched by reagents added from the cytosolic side of the ER but they become accessible to quenching reagents that gained access to the lumen of the ER (Crowley et al., 1993; 1994). This suggests that after the transition from loose to tight ribosome binding to the ER membrane one continuous channel exists from the peptidyl-transferase center in the ribosome to the lumen of the ER.

Only ribosome-nascent chain complexes containing a functional signal sequence undergo the transformation from a loose to a tight state of ribosome-membrane interaction (Jungnickel and Rapoport, 1995). Therefore this transition comprises the following events:

- Recognition of the signal sequence by the translocation channel
- A shift to high salt resistant binding of the ribosome to the Sec61p complex
- Closing of the ribosome-membrane junction to the cytosol
- Opening of the channel towards the lumen of the endoplasmic reticulum

All events are triggered by binding of the signal sequence to the Sec61p complex. This occurs when the nascent chain has reached a length such that the signal sequence can interact productively with the Sec61 channel. It has been suggested that the nascent chain inserts into the translocation channel as a loop with its N-terminus located in the cytosol (see Figure 3B; Shaw et al., 1988; Mothes et al., 1994). The critical length of the nascent chain for signal sequence recognition by the Sec61p complex varies slightly for different substrates (Mothes et al., 1998), possibly reflecting differences in the length of the signal peptide and of the hydrophobic core.

So far, the molecular mechanisms are unknown by which the transition from loose to tight ribosome binding occurs. The insertion of a nascent chain into the translocation channel could cause a conformational change that increases the strength of the ribosome-channel interaction, or the chain could merely provide an additional, stabilizing link. The 3D map of the yeast nontranslating ribosome bound to the yeast Sec61p complex (Beckmann et al., 1997) most likely visualizes the ribosome-channel junction in the loose binding stage. In this structure, a large gap is seen between the ribosome and the Sec61p complex. However, the biochemical data (Crowley et al., 1994) might suggest that in the tight binding state the ribosome-membrane interaction would become much more intimate and that a continuous sealing exist around the ribosome-membrane junction. Therefore, one of the most exciting questions asked in this thesis is whether structural differences can be detected in 3D maps of ribosome-channel complexes with and without a nascent chain.

1.6. The Signal Sequence in the Translocation Channel

Using site-specific photocrosslinking, the environment of signal sequences in translocation intermediates after signal sequence recognition by the Sec61p complex has been mapped (Plath et al., 1998). The signal sequence contacts Sec61α and seems to be precisely oriented with respect to the Sec61p complex. The crosslinking pattern suggests that in the translocon the signal sequence adopts an α-helical structure. The signal sequence has been shown to be in close proximity to proteinaceous components of the translocation channel as well as to lipid molecules,

indicating that it is located at the interface of protein channel and lipid phase (Mothes et al., 1998). A similar study of signal sequence recognition in posttranslational translocation in S.cerevisiae supported these results (Plath et al., 1998). In addition, these data revealed that the membrane spanning domains 2 and 7 of the yeast Sec61α homologue face the binding site and sandwich the α helix formed by the signal sequence.

In the mammalian cell, another component of the translocon, the translocating chain associated membrane (TRAM) protein, interacts with the signal sequence (Görlich et al., 1992b; High et al., 1993; Plath et al., 1998). It seems that TRAM contacts one side of the signal sequence when it is inserted into the translocation channel as an α-helix. Interestingly, the same side is interacting with Sec62p in the posttranslational translocation pathway in yeast (Plath et al., 1998).
Experiments employing reconstituted proteoliposomes containing purified membrane proteins have shown that TRAM is essential for translocation of most secretory and membrane proteins (Görlich et al., 1992b; Görlich and Rapoport, 1993; Voigt et al., 1996). However, the function of TRAM in protein translocation across the membrane of the ER remains elusive. TRAM dependence or independence is conferred by the signal sequence. Proteins containing a long and very polar N-region and a long hydrophobic core rich in leucine and valine residues are generally TRAM-independent (Voigt et al., 1996).

Another component of the translocon that has been found in proximity to the signal sequence (Wiedmann et al., 1987) and to mature regions of the nascent chain during translocation (Krieg et al., 1989; Wiedmann et al., 1989) is the heterotetrameric translocon associated protein (TRAP) complex. TRAP has also been shown to be tightly associated to the membrane bound ribosome (Görlich et al., 1992a). Although the TRAP complex seems to be an integral part of the translocon it is not essential for translocation of all proteins tested so far (Migliaccio et al., 1992; Görlich and Rapoport, 1993). For the secretory protein preprolactin it has been demonstrated that TRAP does not even stimulate translocation (Görlich and Rapoport, 1993).

1.7. Later Stages of Cotranslational Protein Translocation Across the ER Membrane

As the nascent chain is elongated further, the signal sequence is cleaved off by the signal peptidase complex at a recognition site following the hydrophobic core (Evans et al., 1986). After cleavage, the signal peptide remains in the lipid bilayer where it is subsequently processed by a signal peptide peptidase. Fragments of the signal sequence are then released into the cytosol (Lyko et al., 1995).
The oligosaccharyl transferase complex transfers carbohydrate chains from dolicholphosphate onto glycosylation sites of the nascent chain (Kelleher et al., 1992). Other ER resident proteins presumably interacting with newly translocated polypeptides are protein disulfide isomerase (PDI), which facilitates the formation of disulfide bonds, prolylpeptidyl cis/trans isomerase, which promotes prolyl cis/trans isomerization, and chaperones in the ER lumen, assisting in folding of translocated polypeptides.

In contrast to early stages of translocation, which are well characterized, little is known about how translocation is terminated. It has been suggested that release of the nascent chain and dissociation of the ribosome from the translocation site are necessary for closing of the translocation channel (Simon and Blobel, 1991). Yet, ring-like structures formed by the Sec61p complex were observed in microsomes that had been treated with puromycin under high salt conditions to release nascent chains and remove membrane bound ribosomes (PKRM; Hanein et al., 1996). Thus, Sec61p channels may remain assembled in a closed conformation after termination of translocation and release of the ribosome. Alternatively, puromycin-induced termination may not result in disassembly of the channel that may occur under physiological conditions. Strikingly, solubilization of PKRM with digitonin yields intact Sec61p complexes that can promote protease-resistant insertion of short translation intermediates (Mothes et al., 1998). Additional membrane proteins have been shown to be tightly associated with membrane bound ribosomes after detergent solubilization of rough microsomes (Görlich et al., 1992a) suggesting that they may be part of a larger translocation complex that assembles for each round of translocation and disassembles after translocation has been terminated.

1.8. Biogenesis of Integral Membrane Proteins

Membrane proteins destined to reside in the plasma membrane, lysosomes, the Golgi network or the ER also follow the secretory pathway (for review see Hegde and Lingappa, 1997; Matlack et al., 1998) and use the same translocation machinery as soluble proteins (Görlich and Rapoport, 1993; Oliver et al., 1995). First, the precursors are targeted to the ER membrane as ribosome-nascent chain complexes. For targeting, membrane proteins may use a cleavable signal peptide or a noncleavable signal anchor. Signal anchors are generally longer and more hydrophobic than signal peptides (for review see von Heijne and Manoil, 1990). Furthermore, they lack the recognition site for the signal peptidase. SRP interacts with both signal anchors and signal sequences of integral membrane proteins. After targeting, nascent membrane proteins are translocated across the ER membrane and their one or more membrane spanning domains are integrated into the phospholipid bilayer in the correct orientation.

It is not clear yet at which point a membrane anchor is transferred from the proteinaceous channel to the lipid phase. It has been suggested that membrane spanning domains either remain in a proteinaceous environment until termination of translation and release of the nascent chain from the ribosome (Borel and Simon, 1996; Do et al., 1996) or that membrane anchors contact phospholipids in an early phase of translocation, suggesting that even during translocation the Sec61p channel opens laterally towards the lipid phase (Martoglio et al., 1995; Mothes et al., 1997).
Another open question is how the translocation channel is gated during biogenesis of an integral membrane protein. According to one study, the channel may be closed towards the ER lumen and opened towards the cytosol while the membrane anchor is still buried within the translating ribosome (Liao et al., 1997), implying that the ribosome can recognize a membrane anchor and induce a conformational change of the translocation channel. This would be very different from the gating of the channel that is induced by binding of the signal sequence to the Sec61p complex (Crowley et al., 1994; Jungnickel and Rapoport, 1995).

1.9. Regulation of Ribosome-Binding to the Sec61p complex

It has been shown that nontranslating ribosomes have an intrinsic affinity for microsomal membranes (Borgese et al., 1974; Kalies et al., 1994). At the ER membrane, the Sec61p complex functions as the ribosome receptor (Görlich et al., 1992; Kalies et al., 1994). It has also been shown that ribosome-nascent chain complexes (RNCs) can be targeted to the ER membrane by the interaction of ribosomes and translocation sites alone (Lauring et al., 1995b; Jungnickel and Rapoport, 1995). If all ribosomes, independent of the nature or presence of a nascent chain, can bind to translocation sites at the ER membrane, how does efficient targeting occur for RNCs carrying a signal sequence?
Recently, a model invoking a cytosolic inhibitor of ribosome binding to the Sec61p complex has been proposed (Wiedmann et al., 1994). This model suggests that the inhibitor, nascent polypeptide-associated complex (NAC) binds to nascent chains emerging from the ribosome and to nontranslating ribosomes. The presence of NAC on RNCs (Lauring et al., 1995a) or on nontranslating ribosomes (Möller et al., 1998) is thought to prevent binding to SRP and to the ER membrane. In the case of nascent chains with a signal sequence, SRP would be able to compete off NAC by specific binding to the signal sequence (Lauring et al., 1995b), allowing the ribosome-nascent chain complex to be targeted to the membrane.
According to this model, only ribosomes carrying a nascent chain with a signal sequence and bound SRP are targeted to the ER membrane. Signal sequence recognition by the Sec61p complex during subsequent stages of translocation would merely be a double check.

The role of NAC in regulation of ribosome binding to the ER is somewhat controversial. First of all, a substantial population of ribosomes is bound to rough microsomes in a high salt sensitive manner (about 45%, Kalies et al., 1994), indicating that they are not engaged in translocation. It is unlikely that such a large fraction presents ribosome-nascent chain complexes in early, salt-sensitive, stages of translocation. Furthermore, one study affirmed the specificity of SRP for signal sequence-bearing RNCs (see Walter et al., 1981) in the presence and absence of NAC (Powers and Walter, 1996). Besides, all data supporting the NAC model were derived

from in vitro experiments using RNCs that had been washed with a high salt buffer to remove NAC and SRP. It has been shown that these high salt washed RNCs can bind to microsomes in a manner that is independent of SRP and the presence of a signal sequence. Readdition of NAC restored SRP and signal sequence dependent binding. So far, the role of NAC in regulation of ribosome binding to the ER membrane has not been studied under more physiological conditions.
Another argument against the proposed role of NAC in regulation of ribosome targeting comes from experiments in S. cerevisiae. The deletion of NAC homologues in yeast does not result in a growth defect or in a secretion phenotype (Reimann et al., 1999). Instead there are reports that in S. cerevisiae NAC is involved in protein targeting to mitochondria (George et al, 1998; Fünfschilling and Rospert, 1999) and that NAC homologues are functioning as transcription factors for GAL4 (Shi et al., 1995). The function of NAC as a transcriptional co-activator for GAL4/VP-16 was confirmed in mammalian cell lines (Yotov et al., 1998). It was also reported that the α subunit of NAC potentiates c-Jun mediated transcription (Moreau et al., 1998).

One aim of this thesis was to re-evaluate the function of NAC by studying SRP-independent targeting of signal sequence-bearing RNCs in a full translation system. We also wished to determine which role SRP binding to the signal peptide plays and how signal sequence recognition by the Sec61p complex is important in achieving specificity of targeting.

1.10. Aims

The thesis project presented here explores different aspects of initial steps in cotranslational protein translocation.

1. The N-terminal hydrophobic signal sequence is known to bind to the cytosolic signal recognition particle (SRP). Yet, since in the cell there is only one SRP for every 10-100 ribosomes (Walter and Johnson, 1994; Ogg and Walter, 1995) it is conceivable that hydrophobic signal peptides can interact with cytosolic factors other than SRP; either during SRP-independent targeting of RNC to the ER membrane or to prevent aggregation in a hydrophilic milieu before SRP binding. Using a photocrosslinking approach we wanted to probe the cytosolic environment of the signal sequence and identify possible binding partners.

2. All ribosomes, independent of the presence or nature of a nascent chain, can interact with the Sec61p complex. Thus, we wished to address the question how under these conditions specificity in targeting is achieved. We were interested in assessing the role of cytosolic factors in regulation of RNC-binding to the Sec61p complex. In addition, we wanted to compare binding of ribosomes with or without various nascent chains in competition assays.

3. Two stages of ribosome binding to the Sec61p complex are known: an initial loose binding and a tighter binding after insertion of a nascent chain into the translocation channel and subsequent signal sequence recognition. Using single particle cryo electron microscopy, we carried out a structural comparison of ribosome-channel complexes with and without a nascent chain, representing the tight and loose binding state, respectively. We also wanted to compare the structures of the native translocation channel and of the channel formed by the isolated Sec61p complex since it is known that other membrane proteins in addition to the Sec61p complex are part of the translocon.

2. MATERIALS AND METHODS

2.1. Materials

-Chemicals and enzymes were purchased from Sigma-Aldrich, Fisher Scientific, New England Biolabs, Inc., Biorad Laboratories, Promega Corp., Pierce Chemical Company, Amersham Pharmacia Biotech, EM Science, Roche Molecular Biochemicals, Calbiochem Corp. and Avanti Polar Lipids Inc.

-Antibodies were raised in rabbits against peptides of Sec61α (CKEQSEVGSMGALLF), Sec61β (PGPTPSGTNC), TRAPα (CLPRKRAQKRSVGSDE), TRAM (CADSPRNRKEKSS) by BabCo and Cocalico Corp. Antibodies directed against the α subunit of NAC were a gift from Dr. M. Wiedmann, antibodies against the ribosomal protein S26 were a gift from Dr. J. Stahl.

-Truncated mRNAs coding for nascent chains were generated by *in vitro* transcription as described before:

ppl86, ppl59, ppl86Δ13-15	Jungnickel and Rapoport, 1995
ppl132	Mothes et al., 1994
ppl169	Görlich et al., 1992
ppαFt86amb8 to 16, ppαFt86K5	Plath et al., 1998
lep57, lep68, lep70, lepcyt, lepXa	Mothes et al., 1997
Invertase	Voigt et al., 1996
ffl77	Wiedmann et al., 1994

-Photocrosslinking reagents were prepared as described in Görlich et al., 1991 (TDBA-lysyl tRNA) and Martoglio et al., 1995 (TmD-Phe suppressor tRNA).

-Purified NAC was a gift from Dr. M. Wiedmann. Calmodulin purified from bovine brain was purchased from Calbiochem Corp. SRP was purified as described in Walter and Blobel, 1983a.

2.2. Preparation of Microsomes

Rough microsomes (RM) from canine pancreas were prepared as described in Walter and Blobel, 1983b. For preparation of puromycin-high salt treated microsomes (PKRM), a suspension of RM containing 3.5 equivalents (eq.; for definition, see Walter, et al., 1981) per µl was mixed with an equal volume of buffer B (100mM Hepes/KOH pH7.6, 200mM sucrose, 300mM potassium acetate, 10mM magnesium acetate, 3mM dithiothreitol (DTT), 0.2mM GTP, protease inhibitors (10µg/ml leupeptin, 5µg/ml chymostatin, 3µg/ml elastatinal, 1µg/ml pepstatin), 3mM puromycin). After homogenization, the mixture was incubated for 1hr at 0°C, followed by 10 min at 37°C and 10 min at room temperature. The sample was centrifuged in a Beckman TLA100.3 rotor for 30 min at 100,000 rpm at 2°C. The pellet was resuspended at 10 eq./µl in buffer C (50mM Hepes/KOH pH7.6, 500mM sucrose, 800mM CsCl, 15mM magnesium acetate, 3mM DTT, protease inhibitors) and mixed with an equal volume of a buffer identical to buffer C, except that it contained 1.95M sucrose. The total volume was determined and additional CsCl was added to a final concentration of 700mM. The sample was overlaid with 1ml buffer D (50mM Hepes/KOH pH7.6, 800mM sucrose, 700mM CsCl, 15mM magnesium acetate, 3mM DTT, protease inhibitors) and 0.6ml buffer B in a 13x51mm polycarbonate tube. After centrifugation in a TLA100.3 rotor for 1hr at 100,000 rpm at 20°C, the top 0.2ml were discarded and the following 2-2.5ml, which contained the membranes, were collected. This fraction was diluted 1:4 in 50mM Hepes/KOH pH7.6, 1mM DTT, protease inhibitors, and centrifuged in a TLA100.3 rotor for 30 min at 100,000 rpm at 2°C. The pellet was resuspended in buffer A (50mM Hepes/KOH pH7.6, 250mM sucrose) containing 1mM DTT and protease inhibitors. The membranes were washed once or twice by resuspension and centrifugation and finally taken up at 2-3 eq./µl in buffer A containing 1mM DTT. Salt-washed rough microsomes (KRM) were essentially prepared as PKRM, except that the puromycin reaction was omitted and that the flotation of the membranes was carried out at 2°C.

2.3. Purification of Membrane Proteins and Reconstitution into Proteoliposomes

The purification of the SRP receptor and the Sec61p complex as well as their reconstitution into proteoliposomes were carried out as described (Görlich and Rapoport, 1993; Jungnickel and Rapoport, 1995). The concentrations of the SRP receptor and Sec61p complex in the suspensions of proteoliposomes were 0.8-3 eq./µl and 3-8 eq./µl, respectively.

2.4. Preparation of Ribosome-Nascent Chain Complexes

Truncated mRNAs were translated in a wheat germ or reticulocyte lysate system in the presence of ^{35}S-methionine (Jungnickel and Rapoport, 1995). Translation in the wheat germ system was carried out for 13 min at 28°C, followed by addition of 2µM edeine and further incubation for 5 min. Translation in the reticulocyte lysate was carried out for 25 min at 28°C. Where indicated, TmD-Phe suppressor tRNA or TDBA-lysyl tRNA were present during translation.

To isolate RNCs (see also Lauring et al., 1995a) 100µl of the translation mixture were diluted in 900µl of 40mM Hepes/KOH pH7.6, 2mM magnesium acetate, 2mM DTT containing either 150mM potassium acetate (for low salt-washed RNCs) or 500mM potassium acetate (for high salt-washed RNCs). The samples were layered on top of a 1ml cushion containing a low or high salt concentration (40mM Hepes/KOH pH7.6, 0.5M sucrose, 2mM magnesium acetate, 2mM dithiothreitol and either 150mM or 500mM potassium acetate) in a 13x51mm polycarbonate Beckman tube. After centrifugation for 1 hr at 100,000 rpm at 4°C in a TLA100.3 rotor, the ribosome pellets were resuspended at a concentration of approximately 140nM in ribosome buffer (50mM Hepes/KOH pH7.6, 250mM sucrose, 150mM potassium acetate, 2mM magnesium acetate).

For ribosome competition experiments, RNCs produced in a reticulocyte lysate were isolated by centrifugation through a sucrose gradient containing 150mM potassium

acetate, as described for wheat germ RNCs, and incubated with 10mM *N*-ethylmaleimide for 40 min on ice. The reaction was quenched with 50mM DTT.

Mock translation mixtures lacking mRNA and amino acids were prepared and incubated as described above. Salt washed, non-translating ribosomes were isolated from a mock translation mixture as described for the isolation of salt-washed RNCs. The mock translation was divided into a cytosolic and a ribosomal fraction by centrifugation for 1hr at 100,000 rpm in a Beckman TLA100 rotor. The ribosome pellet was resuspended in the original volume in ribosome buffer.

2.5. Ribosome Competition

A translation mixture containing approximately 200fmoles RNCs (determined by measuring the absorption at 260nm and assuming that a solution with 1 A_{260} absorption contains 16nM ribosomes; Hanein et al., 1996) was mixed with either a mock-translation mixture or a translation mixture containing a different population of RNCs in which the concentration of ribosomes was determined in the same manner. When subfractions of mock translations were used in competition experiments, the amounts added were equivalent to the original volume of the mock-translation mixture. Where indicated, SRP (10nM) was added after translation and before addition of the mock-translation mix. After addition of PKRM (0.4 eq. per 5µl final volume) or reconstituted proteoliposomes (1µl per µl of final volume) incubation was carried out for 10 min on ice and 5 min at 28°C. Experiments with isolated RNCs were performed in an analogous manner. Alternatively, the membranes were preincubated with one population of ribosomes or RNCs before addition of the other population.
For competition experiments using the lepXa fragment, RNCs carrying the lepXa chain were first targeted to the ER membrane and then subjected to treatment with factor Xa as described in Mothes et al., 1997.

2.6. Insertion and Translocation Assays

To assay membrane insertion of ppl86, the samples were incubated with 1 volume of 1.5mg/ml proteinase K in ribosome buffer containing 1mM DTT and 8mM magnesium

acetate for 45 min on ice. The proteinase K reaction was stopped by precipitation of the sample with 15% TCA.
Translocation of membrane-targeted nascent chains was induced by incubation of the samples with 1.5mM puromycin for 10 min on ice and 30 min at 37°C.
Targeting assays using flotation of membrane-targeted RNCs in a sucrose gradient were carried out as described (Jungnickel and Rapoport, 1993; 1995). The flotation of the membranes was confirmed by immunoblotting with antibodies against ER membrane proteins.
Alternatively, membrane targeting was assayed by sedimentation of the microsomes through a sucrose cushion. After incubation of RNCs with microsomes, the sample was diluted to 30µl and loaded on top of 200µl of a buffer containing 50mM Hepes/KOH pH7.6, 500mM sucrose, 150mM potassium acetate, 2mM magnesium acetate, 2mM DTT in 7x20mm polycarbonate tubes (Beckman). The samples were centrifuged for 5 min at 55,000 rpm in a Beckman TLS55 rotor at 2°C. The supernatant was precipitated with 15% TCA and both supernatant and pellet fraction were analyzed by SDS-PAGE. Sedimentation of the membranes was monitored by immunoblotting with antibodies against ER membrane proteins.

2.7. Sample Preparation

Most samples were precipitated with trichloroacetic acid and separated in 13.75% or 7.5-17.5% Tris-Glycine polyacrylamide gels or in 12% or 16% Tris-Tricine gels. For experiments using radiolabeled nascent chains, the gels were dried, exposed to Fuji PhosphoImager screens and quantitated using the Fuji BAS1000 software. For immunoblotting, the proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and incubated with antibodies. Subsequently, the blots were developed using an ECL kit.

2.8. Photocrosslinking

RNCs carrying photoreactive groups were generated in the wheat germ or reticulocyte lysate systems by translation in the presence of TmD-Phe suppressor tRNA or TDBA-lysyl tRNA. Where indicated, the RNCs were isolated after translation.

After the RNCs were either targeted to ER membranes as described above or after they were incubated with the denoted cytosolic factors for 5 min on ice and 5 min at 28°C, the samples were irradiated for 10 min on ice. Crosslinks of ppl86 to membrane proteins were analyzed by immunoprecipitation with Sec61α and TRAM antibodies as described (Görlich et al., 1992).

2.9. Preparation of Bovine Pancreatic Cytosol and Purification of Calmodulin

As a source of bovine cytosol, the 100,000 g supernatant of a bovine pancreatic RM preparation was used (see Walter and Blobel, 1993). For purification of calmodulin, the 100,000 g supernatant was filtered (0.45µm pore size) and incubated for 10 min at 95°C. Aggregates were pelleted by an initial centrifugation of the sample for 10 min at 20,800 g at 4°C and a subsequent centrifugation for 10 min at 100,000 rpm at 2°C in a Beckman TLA100.3 rotor. The supernatant was adjusted to 50mM Hepes/KOH pH7.6, 250mM sucrose, 200mM potassium acetate, 2mM magnesium acetate, 2mM DTT and loaded onto DEAE sepharose. After washing of the column, elution was carried out using a salt-gradient (200 to 800mM potassium acetate) in a buffer as above. All fractions and aliquots taken at various steps of the purification procedure were tested in the crosslinking assay and analyzed by SDS-PAGE and Coomassie staining. A protein of the expected size was present in all fractions containing the crosslinking activity and was identified as calmodulin by sequencing.

2.10. Preparation of Ribosome-Channel Complexes for Cryo-Electron Microscopy

mRNA coding for ppl86 was translated in rabbit reticulocyte lysate in the presence of either PKRM or proteoliposomes containing purified SRP receptor and Sec61p complex for 20 min at 27°C. To generate complexes lacking a nascent chain the mRNA was omitted. After translation, 1-acyl-2-[6-{7-nitro-2,1,3,-benzoxadiazole-4-yl amino}-caproyl]-sn-glycero-3-phosphocholine (C6-NBD-PC) in ethanol was added to a final concentration of 1mol% of total phospholipid to follow the fractionation of

membranes under UV light. The translation mixture was adjusted to a final volume of 150µl containing either 2M sucrose (PKRM) or 1.5M sucrose (proteoliposomes), both in 30mM Hepes/KOH pH7.8 and 10mM magnesium acetate buffer. For ribosomes without or with a nascent chain, the buffer contained 100mM or 500mM potassium acetate, respectively. The samples were transferred to a 7x20mm polycarbonate tube (Beckman) treated with 20mg/ml bovine serum albumin for 10 min at room temperature. Thirty µl of the same buffer without sucrose were layered on top. For all experiments several reactions were prepared in parallel. The sample was spun for 1h at 100,000 rpm at 2°C in a Beckman TLA100 rotor. The floated membranes were collected using an UV trans-illuminator. They were dilute approximately 1:3 in a buffer containing 30mM Hepes/KOH pH7.8, 10mM magnesium acetate, 1.5% digitonin (final concentration), and 100mM potassium acetate for proteoliposomes with ribosomes lacking nascent chains or 500mM potassium acetate for proteoliposomes with ribosomes containing nascent chains and for all PKRM samples. After incubation for 15 to 30 min at 4°C with repeated mixing, the samples were centrifuged for 20 min at 100,000 rpm at 2°C in a TLA100 rotor. The pellet was resuspended in 30mM Hepes/KOH pH7.8, 1.5% digitonin, 100mM potassium, and 10mM magnesium acetate. Aliquots were taken at various steps and analyzed by SDS-PAGE and immunoblotting against the α- and β-subunits of the Sec61p complex. Proteinase K treatment was carried out as described above and CTABr-precipitation was done as described in Mothes et al., 1997. All samples were kept at 4°C and frozen for electron cryo-microscopy within 4 hours of preparation.

2.11. Identification of Ribosome-Associated Membrane Proteins

Ribosome-channel complexes derived from KRM were prepared as described above, except that after the final sedimentation the ribosome pellet was resuspended in a buffer containing 50mM HEPES/KOH pH7.8, 1200mM potassium acetate, 10mM magnesium acetate, 1.5% digitonin, 2mM puromycin, 1mM GTP, 2mM DTT and protease inhibitors. The sample was incubated for 30 min at 4°C and for 10 min at 37°C with repeated mixing. Then, the ribosomes were pelleted by sedimentation for 40 min at 70,000 rpm in the TLA100.3 rotor. The supernatant was extracted with Triton X-114 as described in Görlich et al., 1992. The ribosome pellet was

resuspended in ribosome buffer. The samples were separated by SDS-PAGE and analyzed by staining of the gel using Coomassie Brilliant Blue and by immunoblotting with antibodies raised against Sec61α and TRAPα.

2.12. Electron Cryo-Microscopy of Ribosome-Channel Complexes

Suspensions were loaded onto air glow-discharged 300-mesh grids with thin continuous carbon film, supported by a holey carbon mesh. The specimens were blotted and plunged into liquid ethane (Dubochet et al., 1988) in a humid environment at 4°C (>85% relative humidity). A Gatan cryo-transfer system and cryo-holder (model 626-DH) were used to transfer grids into a Philips CM12 transmission electron microscope equipped with a cryo-blade type anti-contaminator and specimen relocation system. All electron micrographs were recorded at 100kV, under minimal dose conditions with a LaB_6 filament, using a defocus range of -1.0 to -1.5µm. Micrographs were recorded at 28,000x magnification on KODAK SO163 film and developed for 12min in full strength D19 developer (KODAK). In some cases, images were recorded with the specimen tilted at 30° using a dynamic defocus spot scan package developed by Dr. I. Tews.

2.13. Three-Dimensional Image Processing and Analysis

Micrographs displaying minimal astigmatism and drift by optical diffraction were chosen for processing. Negatives were digitized on a ZEISS SCAI scanner using a 7µm raster, binned to 14µm (corresponding to 5Å/pixel) and converted to SPIDER format. Image processing was done using the SPIDER software package (Frank et al., 1996). In most cases, particle picking was done by cross-correlating the image against a rotationally averaged frontal view of the yeast ribosome. Feature with a cross-correlation peak higher than 0.5 were windowed from the original micrographs, montaged and interactively de-selected to remove bad particles. In difficult cases, particles were picked interactively from large sections of the original image using WEB (Frank et al., 1996). Pre-centered 2D datasets of ribosome-channel complexes were first aligned against the corresponding ribosome model truncated at 50Å resolution, using Radon alignment methods (Radermacher, 1994). Three alignment

cycles with unshifted images were calculated by updating the 3D reference from the previous cycle (angular refinement). Finally, the original images were shifted according to the previously refined translations and aligned for three more cycles, allowing both angular and translational refinement in 3D. Final 3D maps were generated using R-weighted back-projection. The resolution in each data set was estimated as described in table 4, and is lower than that imposed by the first node of CTF. We minimized the effects of the CTF by Fermi low-pass filtering each final 3D map to the estimated resolution. It should be noted that a gap between the ribosome and the channel was seen even when all frontal and similarly oriented views, for which CTF fringes are most pronounced, were excluded from the 3D analysis.
The threshold representing 100% of the ribosomal volume was chosen on the basis of calculated and experimentally measured partial specific volumes and the known mass of ribosomal protein and RNA. The 100% ribosomal volume used in this work was $4x10^6 Å^3$.
Statistical 3D maps were computed by randomly breaking the datasets into subsets containing 500 particles (without image repetition), and generating the R-weighted 3D maps. Each series of maps was used without scaling to produce an averaged 3D volume and a second volume containing the variance. The ratio of the average to the variance was interpreted as a measure of statistical confidence using the Student′s t-test (Tractenberg and DeRosier, 1985).

3. RESULTS

3.1. Calmodulin Interacts with Signal Peptides

The synthesis of proteins following the secretory pathway is initiated in the cytosol. Newly translated signal peptides are bound by the signal recognition particle (SRP) which subsequently assists in targeting of the ribosome-nascent chain complex to the membrane of the endoplasmic reticulum (ER; for review, see Walter and Johnson, 1994). SRP seems to be the most important cytosolic interaction partner of signal peptides. It has been suggested that SRP is constantly cycling on and off ribosomes while scanning the nascent chain for a signal sequence (Ogg and Walter, 1995). Yet, the ratio of SRP to translating ribosomes in the cell has been estimated to be 1:10 to 1:100 (Ogg and Walter, 1995), implying that an emerging signal sequence may not always be bound by SRP. Since the hydrophobic signal sequence enters the hydrophilic environment of the cytosol, it seems likely that other cytosolic factors can also interact with it. To test this hypothesis we carried out a photocrosslinking study with translation intermediates of the secretory protein preprolactin.

Ribosome-bound translation intermediates of a defined length were generated by *in vitro* translation of truncated mRNA lacking a stop codon (Perara et al., 1986; Mueckler and Lodish, 1986). The translation products were labeled with ^{35}S-methionine and analyzed by SDS-PAGE.
To probe the environment of translation intermediates, modified lysines carrying a photoreactive crosslinker were incorporated into nascent chains at positions where normally lysines would occur. This was done by adding modified lysyl-tRNAs (TDBA-lysyl-tRNA) to the translation reaction (Görlich et al., 1991). Upon irradiation with UV light, the crosslinker is activated to react with molecules in the immediate vicinity (Figure 4; Kurzchalia et al., 1986; Görlich et al., 1992a,b). Proteins in close proximity to the nascent chain were covalently linked to the ribosome-nascent chain complex. The crosslinked product was detected by a shift in molecular weight of the nascent chain.

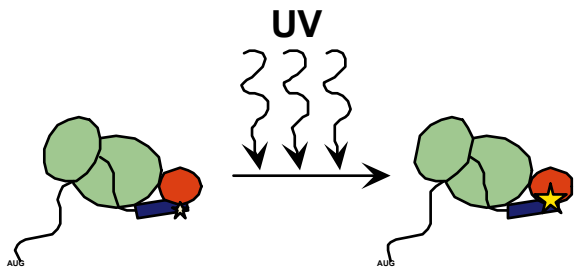

Figure 4: Photoreactive crosslinkers in the nascent polypeptide chain are used to probe the environment of the signal sequence. A modified lysine carrying a photoreactive TDBA group is incorporated into the signal sequence during translation (left). After irradiation with UV light, the nascent chain is covalently linked to proteins (shown in red) in close proximity to the signal sequence (right). Due to an increased molecular weight, the crosslinking product can be detected after the sample has been separated by SDS-PAGE.

When microsomes were added to translation intermediates, the ribosomes were bound to the membrane and the nascent chain was able to interact with the translocation channel (Perara et al., 1986; Connolly et al., 1989). As a result, stable translocation intermediates were created representing distinct stages of protein translocation across the ER membrane depending on the length of the nascent chain.

In order to probe the cytosolic environment of signal peptides, translation intermediates of preprolactin comprising the N-terminal 86 amino acids (ppl86) were generated in a wheat germ system containing very little endogenous SRP. Ribosome-bound translation products were separated from the translation reaction by sedimentation through a sucrose cushion (Jungnickel and Rapoport, 1995). The ppl86 chain has lysines with photoreactive crosslinkers in positions 4, 9, 72 and 78. Since amino acids 72 and 78 are buried within the ribosome, only the two lysine residues in the signal sequence, K4 and K9, could give rise to crosslinks to potential cytosolic interaction partners (Kurzchalia et al., 1986).
When wheat germ cytosol was readded to the translation intermediates, a major crosslink appeared after UV irradiation (Figure 5, lane 3, marked by a star). The interacting partner had an approximate molecular weight of 17kDa. In the absence of cytosol or without irradiation no crosslink was seen (Figure 5, lanes 1 and 2).
A mutant ppl86 chain that has three hydrophobic amino acids deleted from its signal sequence (ppl86Δ13-15; Jungnickel and Rapoport, 1995) did not give rise to a

crosslink to the 17kDa protein (data not shown). This suggests that the interaction with the 17kDa protein depends on a functional signal sequence.

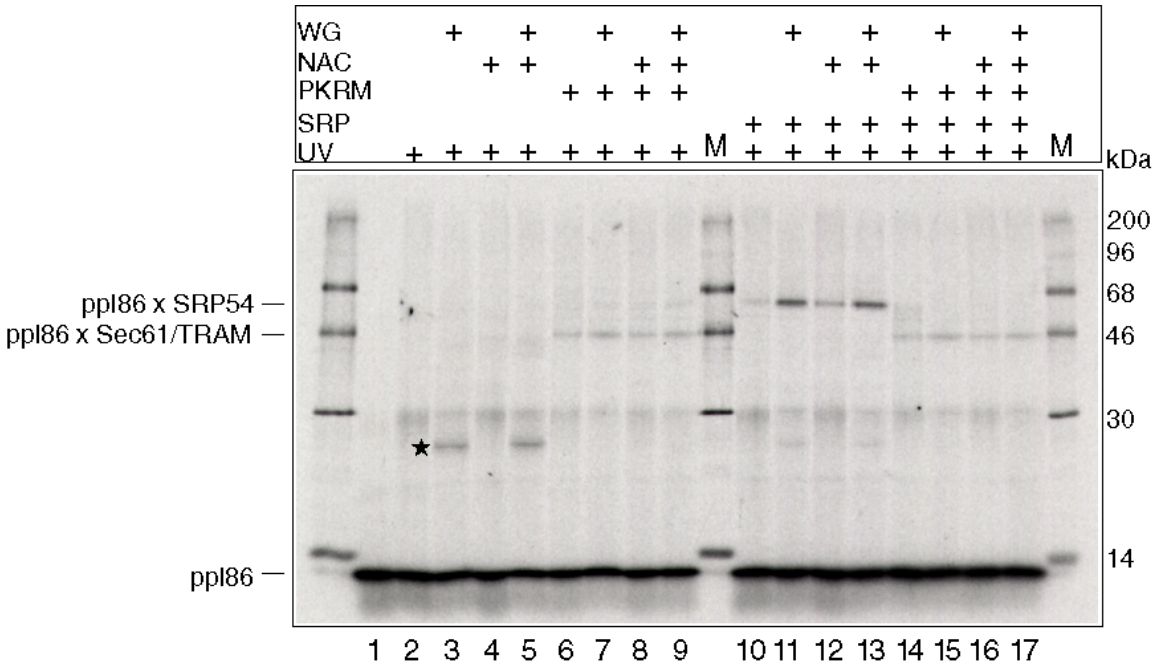

Figure 5: Crosslinking of the ppl86 chain to a cytosolic protein of about 17kDa. Ribosomes carrying the first 86 amino acids of the secretory protein preprolactin were generated in a wheat germ translation system. After translation, the RNCs were isolated by centrifugation through a sucrose cushion. The isolated RNCs were incubated with the following components: wheat germ extract (WG, lanes 3, 5, 7, 9, 11, 13, 15 and 17); purified nascent polypeptide-associated complex (NAC, lanes 4, 5, 8, 9, 12, 13, 16 and 17); microsomes (PKRM, lanes 6 to 9 and lanes 14 to 17); purified SRP (SRP, lanes 10 to 17). After incubation, the samples were subjected to UV irradiation and analyzed by SDS-PAGE. ppl86 x SRP54 indicates crosslinks of the nascent chain to the 54kDa subunit of SRP; ppl86 x Sec61p/TRAM points to crosslinks of ppl86 to the Sec61p complex and the TRAM protein. Lane 1 shows a control sample without irradiation, lane 2 shows a sample that was incubated in the absence of additional factors.

Next, we tested whether signal sequence binding by the 17kDa protein can be competed by other factors. The nascent polypeptide-associated complex (NAC) has been reported to interact with both ribosomes (Möller et al., 1998) and with ribosome-nascent chain complexes (RNC) with or without a signal sequence (Wiedmann et al., 1994). When purified NAC was added to isolated ppl86 translation intermediates, no crosslinks were seen (Figure 5, lane 4). In the presence of both purified NAC and wheat germ cytosol, the 17kDa protein remained bound to the nascent chain (lane 5). In contrast, the intensity of the crosslink of ppl86 to the 17kDa protein was reduced dramatically when purified SRP was added (lane 11 vs. lane 3). Instead, a crosslink to the 54kDa subunit of SRP appeared (lane 11, marked ppl86 x SRP54). When microsomes were present (Figure 5, lanes 6 to 9 and 14 to

17), both the crosslinks to the unknown 17kDa protein (lane 7 vs. lane 3) and to SRP54 (lanes 14 to 17 vs. lanes 10 to 13) disappeared. Instead, crosslinking products to components of the translocation machinery were seen (marked ppl86 x Sec61/TRAM; see also Jungnickel and Rapoport, 1995), suggesting that the nascent chain inserted properly into the translocation channel.
These data indicate that the interaction of the 17kDa protein with the signal peptide can be competed by SRP and ER membranes.

We then used ion exchange chromatography to identify the binding partner of the preprolactin signal sequence. Since a crosslink to a protein of similar size was also seen with other cytosolic extracts (see below) we chose bovine pancreas as a source of cytosol for purification of the crosslinking activity. Using the photocrosslinking assay to monitor the purification process, we isolated the 17kDa protein in a simple two step procedure:
1. Incubation of the cytosol at 95°C and sedimentation of aggregated material
The 17kDa protein proved to be very thermostable and remained soluble (data not shown).
2. Ion-exchange chromatography using DEAE sepharose
The crosslinking activity was bound to the column in the presence of 200mM potassium acetate (data not shown). Elution was carried out with a potassium acetate gradient ranging from 200 to 800mM and fractions were collected. Starting at a concentration of about 500mM potassium acetate, a protein of the expected size was eluted from the DEAE sepharose (Figure 6A, fraction 9 to 12; marked by a star). All fractions containing this protein showed a high crosslinking activity, indicating that it was indeed the interaction partner of the signal sequence (Figure 6B, lanes 8 to 11; the crosslinked product is indicated by a star).

The protein was sequenced and identified as calmodulin. We confirmed that calmodulin can bind signal sequences by testing commercially available calmodulin (purified from bovine brain) in the photocrosslinking assay (Figure 7). A crosslink of the expected size was visible (marked ppl86 x CaM) in the presence of either bovine brain calmodulin (lane 3), wheat germ extract (lane 4), bovine pancreatic cytosol (lane 5) or calmodulin purified from this source (lane 6).

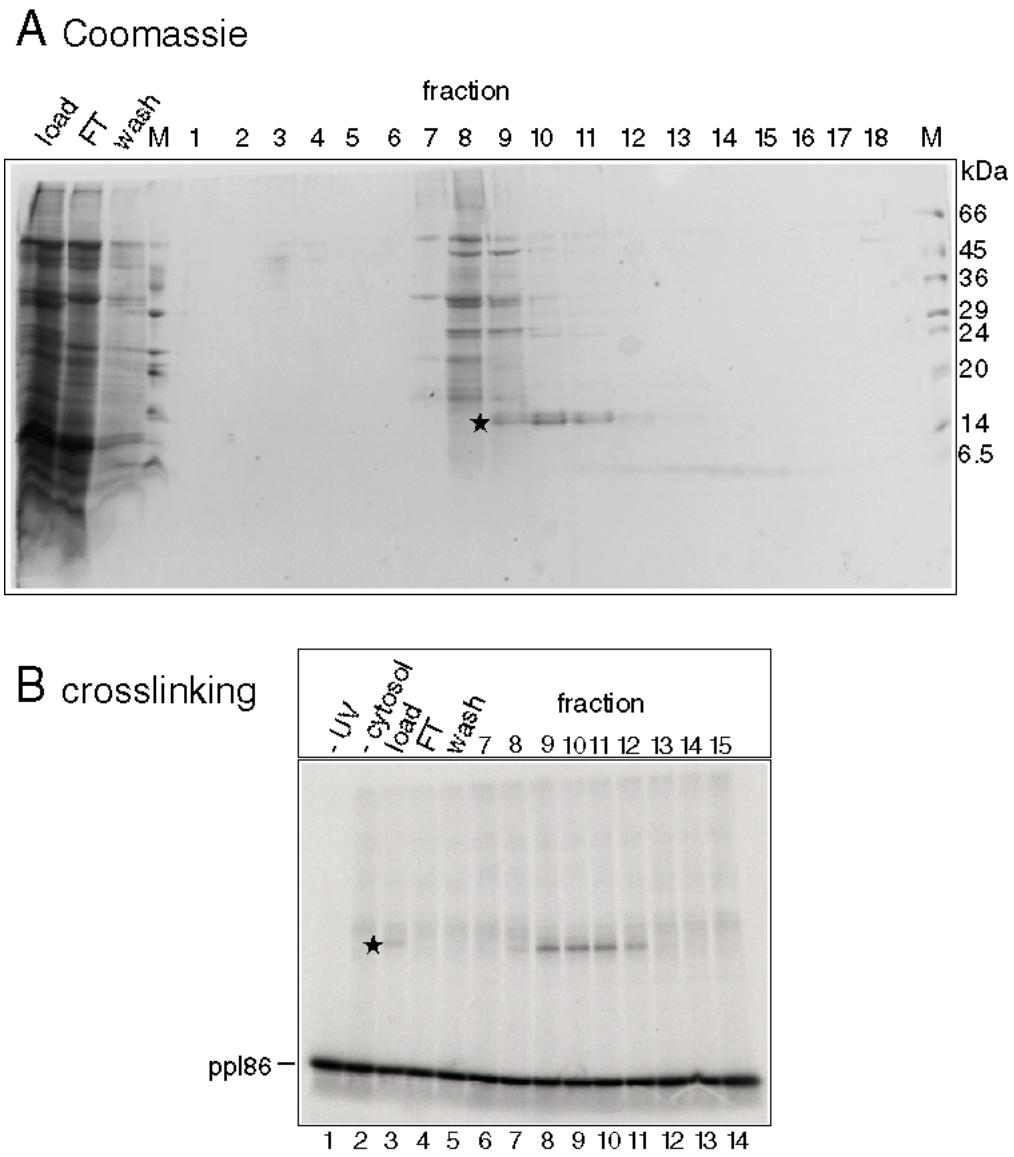

Figure 6: Purification of calmodulin. A: Cytosolic extract from bovine pancreas was boiled for 10 min at 95°C and aggregates were removed by centrifugation. Proteins in the supernatant were bound to DEAE-sepharose and, after washing of the column, were eluted with a potassium acetate gradient ranging from 200mM to 800mM. Aliquots of the collected fractions (1 to 18), of load, flow through (FT) and wash were precipitated with 15% TCA, separated by SDS-PAGE and analyzed by staining with Coomassie Brilliant Blue. The star indicates the protein that was identified as calmodulin. B: Aliquots of DEAE-elution fractions, of load, flow through (FT) and wash were tested for crosslinking activity using the 86mer of ppl. The star denotes the position of the crosslink to calmodulin; ppl86 marks the position of the nascent chain.

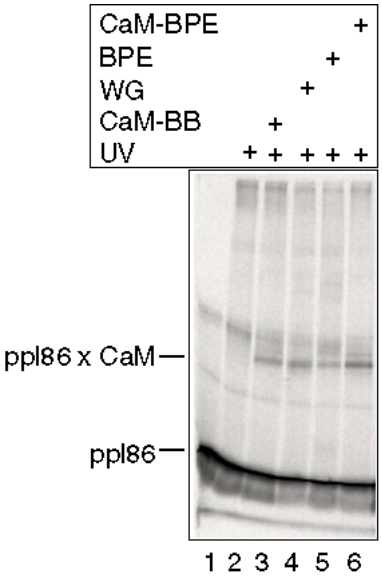

Figure 7: Calmodulin from different sources binds to the signal sequence of ppl86. RNCs of ppl86 were produced in a wheat germ system and isolated by sedimentation through a sucrose cushion. After isolation, the sample was supplemented with either commercially available calmodulin purified from bovine brain (CaM-BB, lane 3), wheat germ extract (WG, lane 4), bovine pancreatic extract (BPE, lane 5) or calmodulin purified from a bovine pancreatic extract (CaM-BPE, lane 6). Subsequently, the samples were irradiated with UV light and analyzed by SDS-PAGE. ppl86 x CaM indicates crosslinks of the ppl86 chain to calmodulin. Samples not subjected to irradiation or incubated in the absence of additional factors are shown in lanes 1 and 2, respectively.

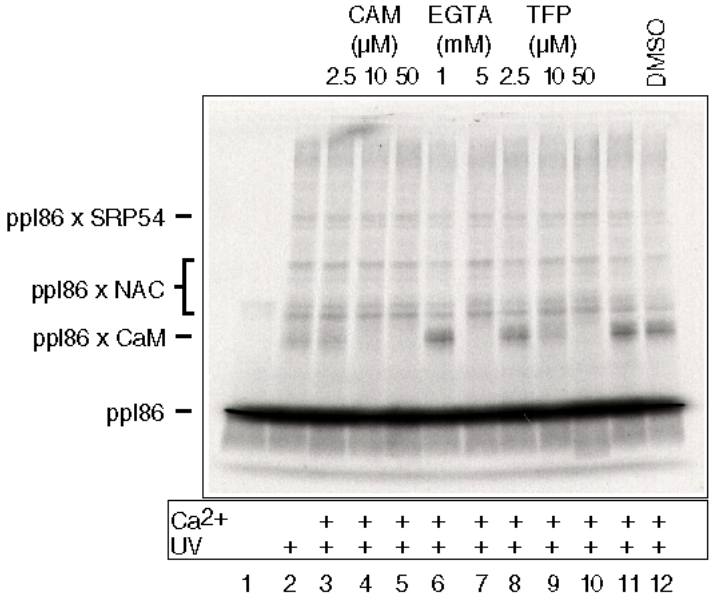

Figure 8: Calmodulin binding to the signal sequence of preprolactin is dependent on Ca^{2+} and sensitive to calmodulin inhibitors. In a wheat germ translation system, RNCs of ppl86 were assembled and subjected to UV irradiation after incubation with a variety of factors. A non-irradiated control sample is shown in lane 1. 1mM $CaCl_2$ was added to all samples except for samples 1 and 2 (lanes 1 and 2). Samples depicted in lanes 3 to 5 were supplemented with increasing concentrations of calmidazolium chloride (CAM), whereas increasing amounts of trifluoperazine (TFP) were added to the samples shown in lanes 8 to 10. In addition, 1mM or 5mM EGTA were used to deplete Ca^{2+} (lanes 6 and 7, respectively). SDS-PAGE was used to separate crosslinked products and nascent chains that had not been linked to other proteins. Crosslinks of ppl86 to calmodulin are indicated by ppl86 x CaM, crosslinks to other cytosolic factors by ppl86 x NAC and ppl86 x SRP.

It has been shown that substrate binding by calmodulin is dependent on Ca^{2+} (James et al., 1995) and can be repressed by specific inhibitors. Therefore, we studied the effect of two frequently used calmodulin inhibitors (calmidazolium chloride and trifluoperazine; Cook et al., 1994; Uemura and Taketomi, 1995) and of the Ca^{2+} chelator EGTA on signal sequence binding by calmodulin. For this and the following experiments, crosslinking was carried out with RNCs that had not been isolated by sedimentation through a sucrose cushion. Instead, the wheat germ translation system, which had been used to generate the RNCs, was supplemented with additional factors after translation.
Upon UV irradiation in the full translation system (Figure 8, lane 2 vs. lane 1), a weaker crosslink to calmodulin was seen (marked ppl86 x CaM). Furthermore, crosslinks to NAC (marked ppl86 x NAC) and residual wheat germ SRP (marked ppl86 x SRP54) were detected. Addition of 1mM Ca^{2+} dramatically increased the amount of nascent chain interacting with calmodulin (lane 11 vs. lane 2). The presence of 1mM EGTA did not have a pronounced effect (lane 6). However, when 5mM EGTA were added, the crosslinks to calmodulin disappeared completely (lane 7). This suggests that calmodulin binding to the signal sequence depends on Ca^{2+}. Both calmodulin inhibitors tested, calmidazolium chloride (Figure 8, CAM) and trifluoperazine (Figure 8, TFP), also prevented the interaction of calmodulin with the signal sequence of preprolactin (CAM, lanes 3 to 5 and TFP, lanes 8 to 10). The addition of DMSO, the solvent of the inhibitors, did not have an effect (Figure 8, lane 12).

For SRP it has been shown that chain elongation renders the signal sequence incompetent for binding to SRP54 (Siegel and Walter, 1988). This is probably due to increased folding of the nascent chain and reflects a translocation-incompetent stage of ribosome-nascent chain complexes. Using longer translation intermediates of preprolactin we tested whether the calmodulin/signal sequence interaction was also dependent on the length of the nascent chain. Indeed, fragments of preprolactin with the N-terminal 132 amino acids showed a reduced crosslinking efficiency to calmodulin when compared to ppl86 (data not shown) and calmodulin binding to a preprolactin chain with 169 amino acids was almost not detectable (data not shown). Thus, binding of calmodulin is decreased upon chain elongation.

To verify that the interaction of signal peptides with calmodulin is not limited to preprolactin we tested other substrates. Using the photocrosslinking approach, calmodulin binding was demonstrated for both the signal sequence of the secretory protein preinvertase and the signal anchor of the integral membrane protein leader peptidase (data not shown).
On the other hand, no interaction was seen using an 86 amino acid fragment of the secretory protein preproαfactor (ppαF86) with a single modified lysine in position 5. However, calmodulin binding to ppαF86 was readily detectable when the environment of the hydrophobic core of the ppαF signal peptide was tested directly using a different site specific photocrosslinking technique (High et al., 1993; Martoglio et al., 1995). In this approach we employed truncated mRNA with an amber stop codon at the position where a crosslinker was desired. During *in vitro* translation amber tRNA carrying a modified phenylalanine with a photoreactive crosslinker was present. Addition of the modified amber tRNA simultaneously suppressed the termination signal and incorporated a photoreactive group into the nascent chain.

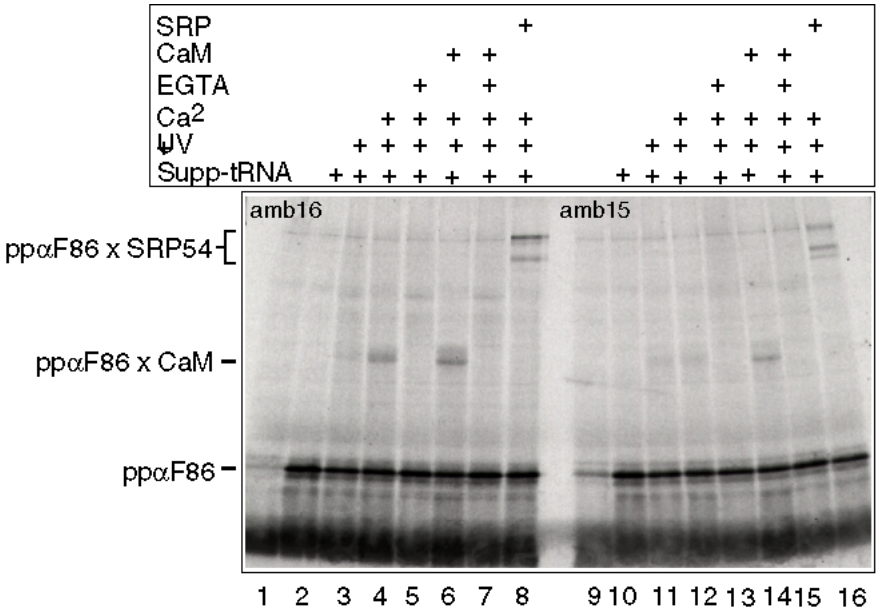

Figure 9: Crosslinking of ppαF86 to calmodulin. Polypeptides corresponding to the 86 amino terminal residues of preproαfactor (ppαF86) were synthesized in wheat germ extract in the presence of amber tRNA carrying a modified phenylalanine with a photoreactive group. The photocrosslinker was placed either in position 15 (amb15, lanes 9 to 16) or in position 16 (amb16, lanes 1 to 8) of the ppαF signal sequence. After translation, samples were supplemented with either 1mM $CaCl_2$ (lanes 4 to 8 and 12 to 16), 5mM EGTA (lanes 5, 7, 13 and 15), purified calmodulin (CaM, lanes 6, 7, 14 and 15) or purified SRP (lanes 8 and 16). The crosslink of the nascent chain to calmodulin is marked as ppαF86 x CaM. ppαF86 x SRP54 indicates the crosslink to the 54kDa subunit of SRP. Lanes 1 and 9 are controls without suppressor-tRNA, lanes 2 and 10 show samples that were not subjected to UV irradiation.

Using the suppressor tRNA technique, it was shown that both the crosslinking efficiency and the electrophoretic mobility of the crosslink to calmodulin were clearly dependent on the position of the photoreactive group. Representative results for ppαF86 with a photocrosslinker in position 15 or 16 are presented in Figure 9 (amb15 and amb16, respectively). In addition, translation intermediates of ppαF86 with crosslinkers either in position 8, 9, 12, 13 or 14 were tested (data not shown).

For both ppαF86amb15 and ppαF86amb16, a fragment corresponding to the first 86 amino acids of preproαfactor appeared upon addition of the amber tRNA to the translation reaction (Figure 9, lanes 2 and 10 vs. lanes 1 and 9, marked ppαF86) indicating that the stop codon was suppressed. When the sample was exposed to UV light and Ca^{2+} had been added, a crosslink to calmodulin was apparent for ppαF86amb16 (Figure 9, lane 4, indicated by ppαF86 x CaM) but only a very weak interaction was detected in the case of ppαF86 amb15 (Figure 9, lane 12). When the reaction was supplemented with additional pure calmodulin, the crosslinking efficiency was increased for ppαF86amb15 (lane 14 vs. lane 12) but remained about the same for ppαF86amb16 (lane 6 vs. lane 4). The crosslink disappeared upon addition of EGTA (lanes 5, 7, 13 and 15), confirming that the binding partner was indeed calmodulin. As expected, in the presence of purified SRP, crosslinks to the 54kDa subunit were visible (lanes 8 and 16, marked by ppαF x SRP54).

Based on the results obtained with preproαfactor, preinvertase and leader peptidase, we conclude that the ability to bind to calmodulin is a general feature of signal sequences. These results suggest that in addition to SRP, calmodulin may be involved in the recognition of signal sequences.

3.2. Regulation of Ribosome Binding to the ER Membrane

It has been shown that all ribosomes, regardless of the presence and nature of a nascent chain can bind to the Sec61p complex (Borgese et al., 1974; Kalies et al., 1994; Lauring et al., 1995b; Jungnickel and Rapoport, 1995). This raises the question of how efficient targeting of RNCs carrying signal sequences can occur.
It has been suggested that the cytosolic factor NAC (nascent polypeptide-associated complex) is an inhibitor of unspecific interactions of ribosomes and RNCs with SRP and the ER membrane (Wiedmann et al., 1994; Lauring et al., 1995a,b; Möller et al., 1998). Binding of SRP to ribosomes synthesizing nascent chains containing signal sequences would compete off NAC, thereby allowing RNCs with a signal sequence to bind to the ER membrane and engage in protein translocation.

However, the role of NAC in the regulation of ribosome targeting to the ER membrane is controversial (see Introduction and Powers and Walter, 1996). Therefore, we decided to reevaluate the function of NAC and the role of SRP in achieving specific targeting of ribosome-nascent chain complexes carrying a signal sequence.

3.2.1. NAC Does Not Prevent Ribosome Binding to the ER Membrane

In previous experiments it has been demonstrated that high salt washed RNCs can be targeted to the ER membrane in an SRP-independent manner (Jungnickel and Rapoport, 1995; Lauring et al., 1995a,b). This was assumed to be due to the removal of NAC by the high salt treatment. We now performed targeting assays in a complete translation system to test whether SRP-independent targeting can occur in the presence of NAC and other cytosolic factors.

To assay SRP-independent targeting of RNCs, a wheat germ system containing low levels of endogenous SRP was used for generation of translation intermediates. Furthermore, wheat germ SRP is known to interact poorly with canine SRP receptor (Prehn et al., 1987). SRP was also absent from the microsomes used in targeting assays because the membranes had been treated with puromycin and high salt

concentrations (PKRM=puromycin/high salt treated microsomes). This procedure not only removes endogenous ribosomes and RNCs but also endogenous SRP.
In addition, we wished to confirm that the wheat germ extract employed here contained physiological concentrations of NAC. Therefore, we compared the amount of NAC in 5 different wheat germ extracts by immunoblotting with antibodies against mammalian NACα (Wiedmann et al., 1994; data not shown). The concentration of NAC in the extracts differed by no more than a factor of three, with the wheat germ extract used for targeting assays containing an intermediate concentration. The absolute concentration of NAC was calculated to be about 0.8µM by comparing it with recombinant mammalian NAC, approximately the same as in reticulocyte lysate. Thus, we conclude that our system is comparable to the system used for establishing the NAC model (Wiedmann et al., 1994; Lauring et al., 1995a,b).

For targeting assays, RNCs carrying the ppl86 fragment were first generated in the wheat germ system, then canine pancreatic microsomes were added. Targeting that has been uncoupled from translation is similar to targeting in a truly cotranslational system (Perara et al., 1986), yet ribosome binding is more efficient since conditions for targeting can be optimized without affecting translation. By using PKRM in our experiments, we also increased the number of available ribosome binding sites.
Targeting of RNCs carrying the ppl86 chain in the absence of SRP, but in the presence of NAC and other cytosolic factors was assessed using three independent assays. To evaluate the efficiency of SRP-independent targeting we carried out parallel experiments using RNCs that had been incubated with purified SRP after translation but before the addition of microsomes.

First, a photocrosslinking experiment using TDBA-lysine was performed to probe the environment of the ppl86 chain after addition of microsomes. RNCs carrying ppl86 have been previously shown to represent a stage of translocation at which the nascent chain is inserted into the translocation channel and the signal sequence is bound by the Sec61p complex (Görlich et al., 1992a,b; Jungnickel and Rapoport, 1995).
In the absence of added SRP, crosslinks of ppl86 to Sec61α and TRAM were observed when PKRM were added (Figure 10, lane 3 vs. lane 2, indicated by ppl86 x

Sec61α/TRAM). The identity of the crosslinked products was confirmed by immunoprecipitations (Figure 10, lane 6 and 7). This shows that even in the presence of NAC and other cytosolic factors, the signal sequence of the nascent chain can contact the translocation channel, indicating that the RNCs are efficiently targeted to the ER.
Crosslinks of the nascent chain to the 54 kDa subunit of SRP were visible when SRP was added (Figure 10, lane 4 and with reduced intensity in lane 5, marked by ppl86 x SRP54) confirming that indeed the signal sequence had been bound by SRP.
In the presence of SRP and microsomes membrane crosslinks appeared, which were similar to those observed in the absence of SRP (Figure 10, lanes 5, 9 and 10). These data suggest that SRP is not essential for efficient targeting of RNCs carrying ppl86 in the presence of NAC and other cytosolic factors

A second approach confirmed these results. We directly tested binding of RNCs to microsomes by employing a flotation assay. RNCs carrying ppl86 were synthesized in wheat germ extract and incubated with PKRM. Next, the membranes were subjected to flotation in a sucrose gradient at physiological salt concentrations. More than 80% of the nascent chain was detected in the membrane fraction both in the absence and presence of SRP (data not shown). Again, these data show that even in the presence of NAC and other cytosolic factors, RNCs can bind to ER membranes in an SRP-independent manner.
To test whether the nascent chain had been inserted into the translocation channel and whether the signal sequence had been bound by the Sec61p complex, the flotation was repeated under high salt conditions (Jungnickel and Rapoport, 1995). Again, more than 80% of the ppl86 chain was floated with the membranes both in the presence and absence of SRP (data not shown). This confirms that for RNCs carrying ppl86, the transition from loose to tight membrane binding can occur independently of SRP even in the presence of NAC and other cytosolic factors.

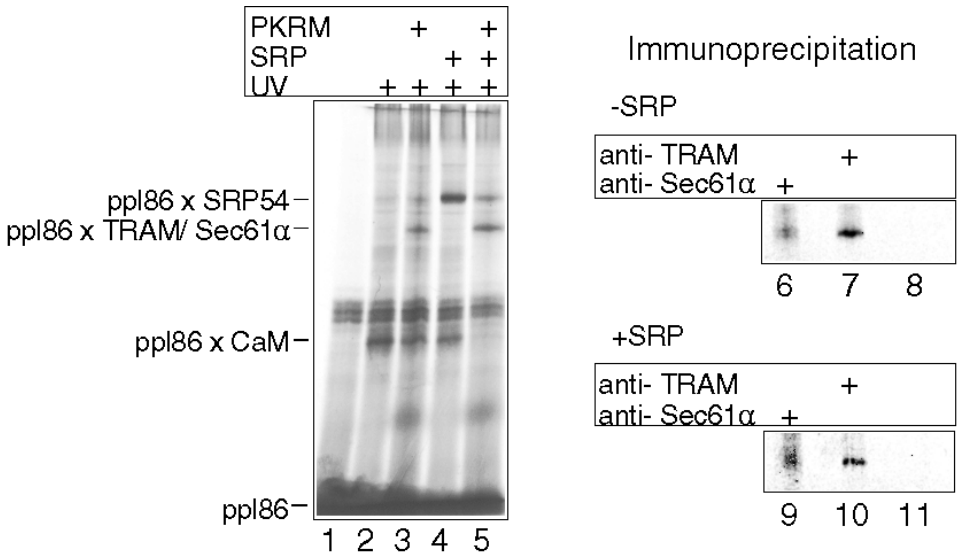

Figure 10: SRP-independent targeting of RNCs in the presence of cytosol. RNCs bearing the ppl86 chain were assembled in a wheat germ translation system containing low levels of endogenous SRP. During translation modified lysines carrying a photoreactive group were incorporated into the nascent chain. Upon irradiation with UV light, crosslinks to calmodulin appeared (lane 2, indicated by ppl86 x CaM). In the presence of purified SRP, crosslinks to the SRP54 subunit were visible (lanes 4 and 5, marked by ppl86 x SRP54). When microsomes were added to the samples (PKRM), crosslinks to Sec61p and the TRAM protein appeared (lanes 3 and 5, denoted by ppl86 x Sec61α/TRAM). Immunoprecipitations of the samples shown in lanes 3 and 5 using antibodies raised against peptides of Sec61α (lanes 6 and 9) or TRAM (lanes 7 and 10) confirmed that the crosslinking products contained these membrane proteins. Lane 1 shows a control sample that was not subjected to UV irradiation. In lanes 8 and 11 mock immunoprecipitations lacking antibodies are shown.

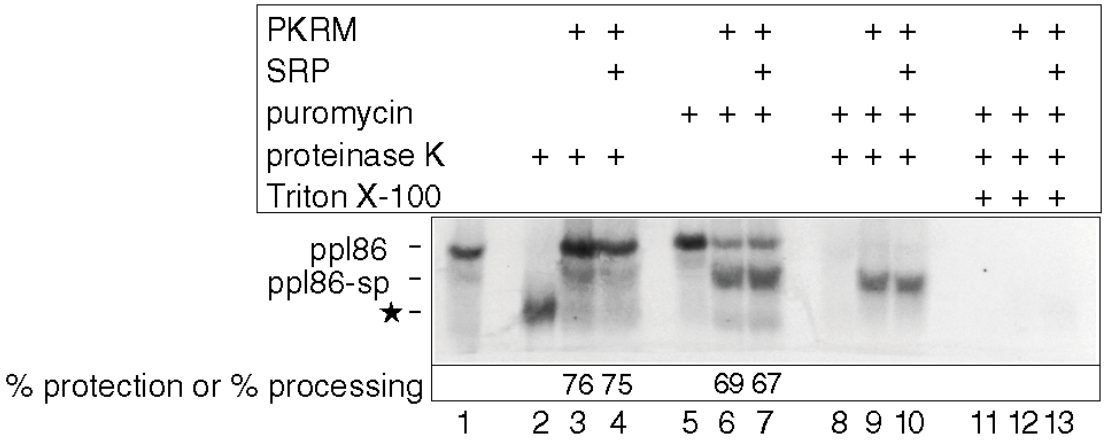

Figure 11: SRP-independent targeting of RNCs in the presence of cytosol. RNCs of ppl86 were assembled in a wheat germ translation system. To some samples purified SRP was added (lanes 4, 7, 10 and 13). After incubation in the absence (lanes 1, 2, 5, 8, 11) or presence (all other lanes) of microsomal membranes (PKRM), targeting and translocation of the nascent chains were evaluated by treatment with proteinase K (lanes 2 to 4) or puromycin (lanes 5 to 7) or by proteinase K digestion after the puromycin reaction in the absence (lanes 8 to 10) or presence of Triton X-100 (lanes 11 to 13). The position of the ppl86 chain is indicated by "ppl86", the star marks the ribosome protected fragment of about 30 residues; the position of the signal sequence-cleaved chain is denoted by ppl86-sp. The amount of nascent chains protected against proteinase K or processed after puromycin treatment is given as % protection or % processing.

We verified our observations using another targeting assay in which the accessibility of nascent chains to protease is determined. Nascent chains whose signal sequence has been bound by the Sec61p complex become protected against proteinase K added from the cytosolic side.

When ppl86 chains synthesized in wheat germ extract were treated with proteinase K in the absence of microsomes, a small fragment of about 30 amino acids was produced (Figure 11, lane 2, marked by a star), representing the C-terminal portion of the nascent chain that is protected by the ribosome. In the presence of membranes, the small fragment largely disappeared and the protected ppl86 chain appeared (Figure 11, indicated by ppl86, compare lanes 3 and 2; for quantitation of targeting efficiency we determined the amount of radioactivity in the ppl86 fragment compared to total protease-protected radioactivity in the sample).

To prove that the protease-protected nascent chains represent translocation intermediates, the polypeptides were released from the ribosome by treatment with puromycin. Properly inserted nascent chains resume translocation after puromycin release; they move into the ER lumen and undergo signal sequence cleavage. Indeed, puromycin-induced signal sequence cleavage was observed (Figure 11, lane 6; ppl86-sp indicates the signal sequence cleaved fragment). The processed ppl86 chains were protected against digestion by proteinase K in the absence of detergent (Figure 11, lane 9) but not in the presence of detergent (Figure 11, lane 12) indicating that the polypeptide had indeed been translocated into the lumen of the vesicles.

In parallel, samples were analyzed that had been supplemented with purified SRP before addition of microsomes (Figure 11, lanes 4, 7, 10 and 13). Quantitation of the results showed that the efficiency of targeting and translocation were identical in the absence and presence of SRP (see Figure 11).

Taken together, these results show that efficient SRP-independent targeting of RNCs and translocation of the nascent chain can occur even in the presence of NAC and other cytosolic factors. Thus, we conclude that NAC does not inhibit ribosome and RNC binding to the ER membrane and therefore, that NAC does not have a function in achieving specificity of targeting.

3.2.2. RNCs Carrying a Signal Sequence Compete with Nontranslating Ribosomes for Membrane-Binding Sites

In addition to challenging the role of NAC in regulation of ribosome binding to the ER membrane, our data also contradict the general view that SRP is essential for membrane targeting of RNCs carrying signal sequences (Walter and Blobel, 1980). This discrepancy might be explained by two major differences between our system and the one used in earlier studies, which have shown that RNCs are targeted to the ER in an SRP-dependent manner. First, earlier data were derived from experiments employing rough microsomes (RM) or salt-washed microsomes (KRM) which contained endogenous ribosomes. Instead, we used microsomes that had been treated with puromycin under high salt conditions to remove endogenous ribosomes and RNCs (PKRM). Thus, in PKRM there are more unoccupied ribosome binding sites available than in the previously used microsomes.
Second, the wheat germ extract used for the targeting assays might show an increased translation efficiency compared to wheat germ extracts used in earlier studies, resulting in a higher fraction of RNCs in the total number of ribosomes. If one assumes that in the absence of SRP all ribosomes, including RNCs carrying a signal sequence, compete for common membrane binding sites, an increased translation efficiency would result in increased targeting efficiency of RNCs. Likewise, a higher number of available ribosome binding sites, such as the ones found in PKRM, would allow more efficient targeting of RNCs in the absence of SRP.
The use of truncated nascent chains in our targeting reaction is not likely to account for the discrepancy in SRP dependence because SRP has previously been found to be required for targeting of these chains (Connolly and Gilmore, 1986).

We wanted to test the assumption that in the absence of SRP, nontranslating ribosomes and RNCs carrying a signal sequence compete for binding to the ER membrane. In addition, we wished to evaluate the role of SRP in the competition. To this end, we simulated a system with low translation efficiency.
As before, RNCs carrying the ppl86 fragment were generated in the wheat germ extract containing low levels of SRP. Next, the RNCs were incubated with PKRM in the presence of increasing amounts of a non-programmed translation mixture (lacking

mRNA and amino acids; mock translation) that was prepared using the same wheat germ extract. Binding of RNCs and insertion of the ppl86 chain was assessed in a protease protection assay (Figure 12). In the presence of mock translation mixture, SRP-independent targeting of ppl86 was indeed greatly reduced. This was the case regardless of whether the membranes had been preincubated with the mock translation (Figure 12, compare lanes 4 to 7 to lane 3; ppl86 marks the position of the ppl86 nascent chain, the star denotes the 30 amino acid fragment) or whether the mock and ppl86 translation reactions were added to the membranes at the same time (data not shown). Thus, low translation efficiency indeed seems to reduce SRP-independent membrane targeting of RNC. This is consistent with the idea that in the absence of SRP, non-translating ribosomes can compete with RNCs for membrane binding sites. Interestingly, the addition of SRP restored efficient targeting of RNCs carrying a signal sequence even when a large excess of non-programmed translation mixture was present (Figure 12, lanes 8 to 12) suggesting that binding of SRP gives RNCs with a signal sequence a competitive advantage over nontranslating ribosomes.

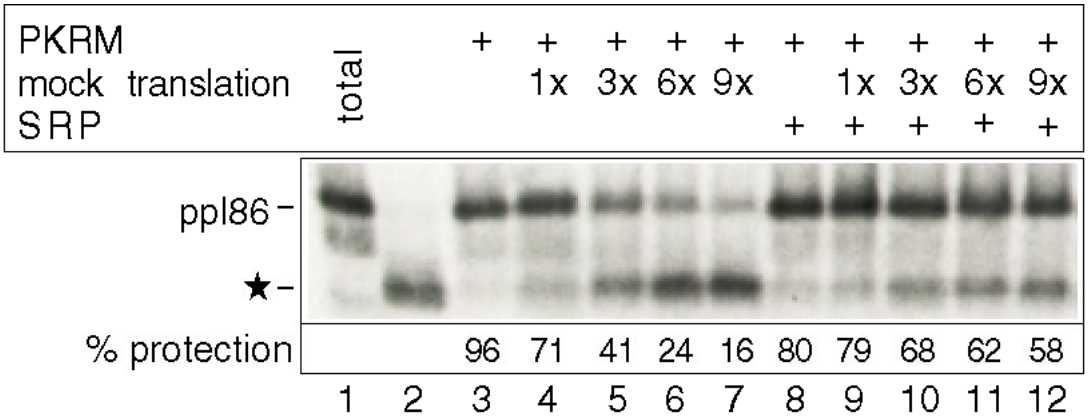

Figure 12: Binding of SRP gives ribosome-nascent chain complexes with a signal sequence a competitive advantage over other ribosomes. RNCs of ppl86 generated in the wheat germ system were targeted to microsomal membranes (PKRM) in the presence of an excess of mock translation mixture (as shown). Where denoted, SRP was added. Targeting efficiency was determined by subsequent digestion with proteinase K (lanes 2 to 12) and is given as % protection. Lane 1 shows a control sample without protease treatment (total). A star indicates the ribosome-protected fragment of the ppl86 chain. ppl86 marks the position of fully protected ppl86.

Next, we tested whether providing additional binding sites can rescue SRP-independent targeting in a system with low translation efficiency. We performed a protease-protection experiment with the 86mer of preprolactin and increasing amounts of PKRM. Targeting of RNCs carrying ppl86 was examined in the absence and presence of a sixfold excess of mock translation mixture. In the absence of mock translation at all membrane concentrations, a substantial part of the nascent chains was resistant to protease treatment, even without added SRP (Figure 13, lane 3 to 5). When mock translation mixture was added, barely any targeting of RNCs was observed in the sample with the lowest membrane concentration (Figure 13, lane 6) whereas almost complete targeting occurred at the highest concentration (Figure 13, lane 8). This indicates that the number of available binding sites indeed determines the extent of SRP-independent targeting in a system with low translation efficiency. Again, addition of SRP restored efficient targeting of RNCs carrying a signal sequence (Figure 13, lanes 12 to 14). As before, these data support the idea that in the absence of SRP, nontranslating ribosomes compete with RNCs for common membrane binding sites, but that RNCs carrying a signal sequence with bound SRP are given an advantage over other ribosomes.

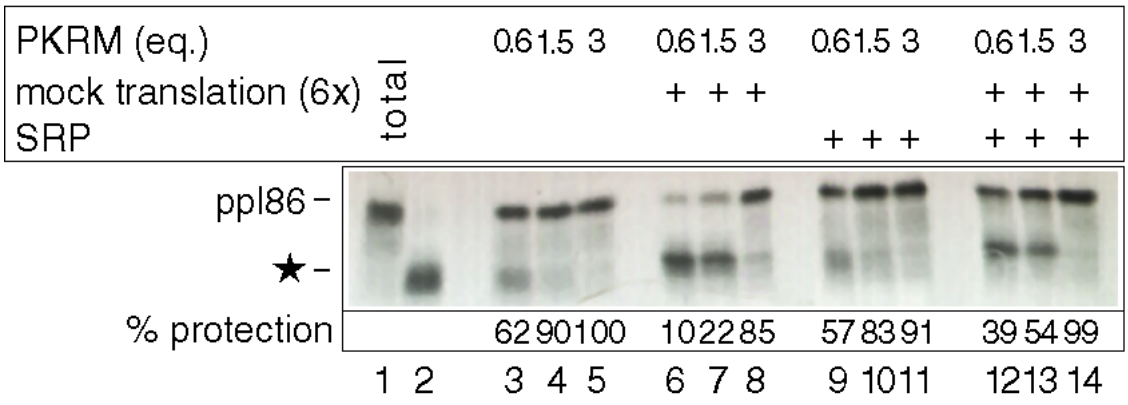

Figure 13: Targeting efficiency depends on the number of available ribosome binding sites. ppl86 was synthesized in a wheat germ system and SRP was added where indicated. In some samples, a sixfold excess of mock translation mixture was present. After incubation with different amounts of PKRM (given in eq.) targeting efficiency was tested by treatment with proteinase K. In lane 1 an undigested sample is shown (total). ppl86 indicates the ppl86 chain. The star marks the fragment protected by the ribosome against protease digestion. The amount of protease-protected ppl86 is given as % protection.

Next, we wished to confirm that nontranslating ribosomes are the cytosolic factors that inhibit SRP-independent targeting of RNCs. Thus, we separated the mock translation mixture into a ribosome pellet and a cytosolic supernatant. As expected, SRP-independent targeting of ppl86 synthesized in wheat germ extract was much reduced when either a sixfold excess of mock translation mixture (Figure 14, lane 4 vs. lane 3) or an equivalent amount of isolated ribosomes were added (Figure 14, lane 6). However, targeting efficiency was not affected by the addition of the cytosolic supernatant (Figure 14, lane 5). In the presence of both the supernatant and pellet fractions, inhibition of SRP-independent targeting was as pronounced as with the complete mock translation mixture (Figure 14, lane 7 vs. lane 4). These data demonstrate that indeed nontranslating ribosomes, but not other cytosolic factors, inhibit SRP-independent targeting of RNCs carrying a signal sequence. Again, the addition of SRP (Figure 14, lanes 9 to 12) allowed efficient targeting of ppl86 even when competing nontranslating ribosomes were present (Figure 14, lanes 9, 11 and 12).

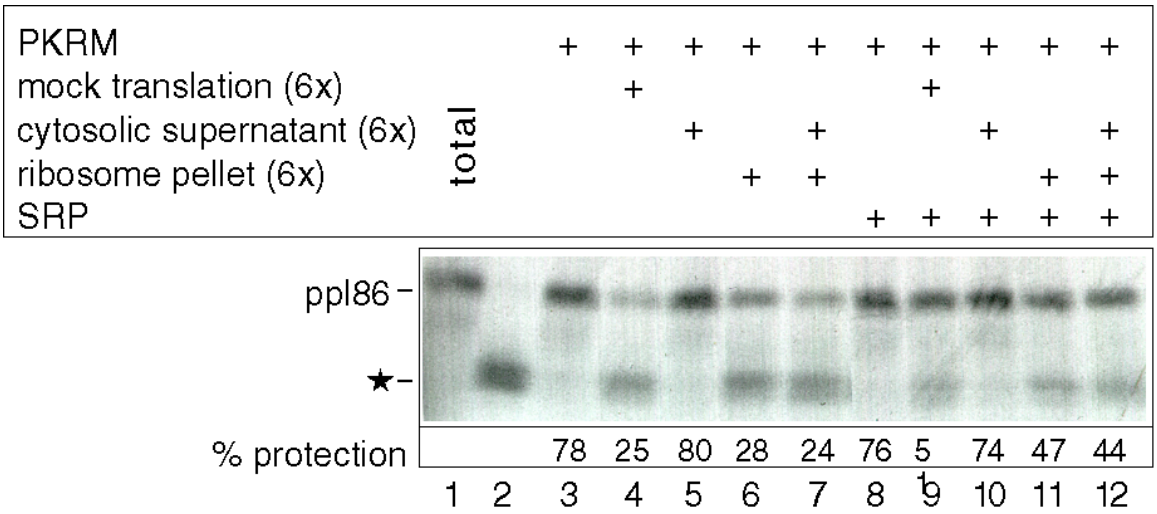

Figure 14: Inhibition of ppl86 targeting by ribosomes. RNCs of ppl86 were assembled in wheat germ extract. Where indicated, a sixfold excess of mock translation mixture was added, or the mock translation was separated into a ribosome pellet and a cytosolic supernatant and equivalent amounts of these fractions were added. Then, the RNCs were bound to PKRM in the absence (lanes 3 to 7) or presence (lanes 8 to 12) of SRP. After targeting, the samples were digested with proteinase K (the amount of protease-protected material is given as % protection). Lane 1 shows undigested ppl86 (total), in lane 2 a sample of protease-treated ppl86 in the absence of microsomes is presented. The star indicates the position of the ribosome-protected fragment of about 30 residues; ppl86 marks the ppl86 chain.

We also performed competition experiments using RNCs carrying ppl86 that had been generated in wheat germ extract and then had been isolated through a sucrose cushion at either physiological salt concentration (150mM, low salt RNCs) or high salt concentration (500mM, high salt RNCs). By doing these experiments, we again

examined the role of NAC in regulation of ribosome targeting. Low and high salt washed RNCs had previously been used to establish the NAC model. Under high salt conditions, ribosome-associated proteins such as NAC, are depleted from the RNCs (Wiedmann et al., 1994), whereas NAC remains bound to RNCs washed at low salt. The presence of NAC in the low salt washed RNCs and its absence from high salt washed RNCs was confirmed by photocrosslinking experiments using RNCs carrying a fragment of 77 amino acids of firefly luciferase (data not shown; see also Wiedmann et al., 1994). Despite the fact that low and high salt RNCs differed in the amount of associated NAC they behaved identically in the targeting assay. A mock translation mixture as well as the ribosome portion of a fractionated mock translation inhibited binding of RNCs to the membrane (data not shown). Addition of the cytosolic supernatant, even though it contained a large amount of NAC, did not affect targeting (data not shown). High salt washed isolated ribosomes also had a strong inhibitory effect (data not shown).

Taken together, these results show that in the absence of SRP, RNCs carrying a signal sequence compete with nontranslating ribosomes for binding to the ER membrane. However, the interaction with SRP gives RNCs with a signal sequence a competitive advantage over other ribosomes. As shown before, NAC, or other cytosolic factors, does not seem to inhibit ribosome binding to the membrane. Based on these conclusions we now understand why efficient targeting in the absence of SRP was observed in the experiments presented in Figure 10 and 11; we had supplied sufficient membrane binding sites for both nontranslating ribosomes and RNCs.

3.2.3. SRP-Independent Targeting in the Reticulocyte Lysate System

We wished to exclude the possibility that our observations on SRP-independent targeting and the lack of inhibition by NAC were restricted to the heterologous system used so far consisting of RNCs produced in wheat germ extract and of canine microsomes. Therefore, we performed targeting reactions with RNCs carrying ppl86 generated in the reticulocyte lysate translation system. Rabbit reticulocyte lysate contains endogenous SRP that interacts well with canine microsomes (Meyer et al., 1982).

After translation in the reticulocyte lysate system, RNCs carrying ppl86 were isolated by sedimentation through a sucrose cushion under physiological salt conditions. Photocrosslinking experiments have shown that under these conditions SRP remains bound to RNCs carrying ppl86 (data not shown). The presence of NAC in RNCs isolated under these conditions was confirmed by photocrosslinking with a 77 amino acid fragment of firefly luciferase (data not shown). The isolated ppl86 RNCs were then targeted to microsomes and subjected to treatment with proteinase K. In the absence of microsomes, most of the nascent chains were degraded to the 30 amino acid fragment that corresponds to the C-terminal portion of the nascent chain buried within the ribosome (Figure 15, lane 2). A small portion of the nascent chain, however, gave a fragment only slightly smaller than ppl86 (lane 2, indicated by an arrow). This may be due to partial protection of the nascent chain by a cytosolic protein. In the presence of microsomes most of the ppl86 was fully protected against proteinase K (Figure 15, lane 3). The addition of competing nontranslating ribosomes did not prevent targeting of RNCs (Figure 15, lanes 4 to 6) as observed before in the wheat germ system in the presence of SRP (see Figure 12).

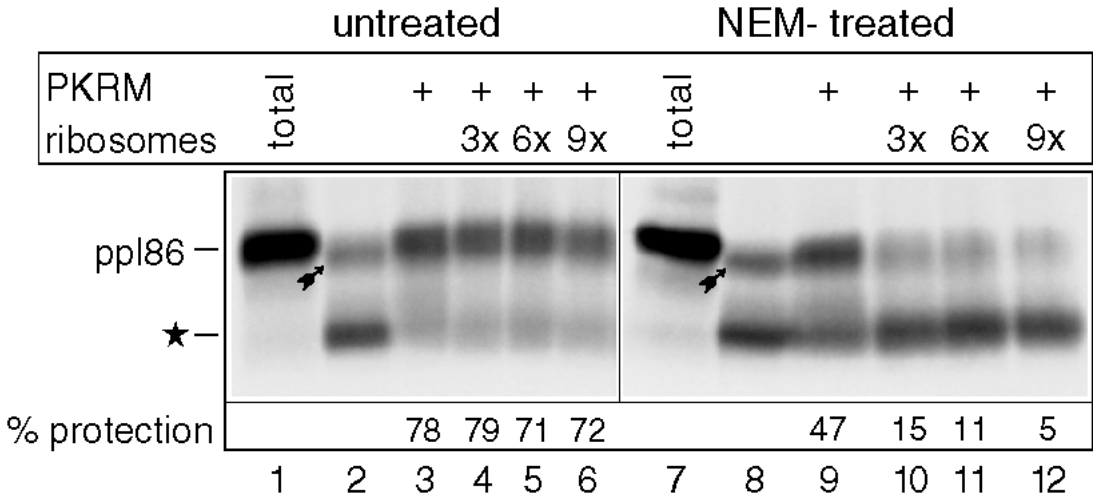

Figure 15: SRP-independent targeting in the reticulocyte lysate system. ppl86 was synthesized in a rabbit reticulocyte lysate translation system and the assembled RNCs were isolated by sedimentation through a sucrose cushion under low salt conditions. One half of the sample was treated with NEM to inactivate SRP, the other half remained untreated. Before incubation of the RNC with PKRM, low salt washed reticulocyte ribosomes, which had also been treated with NEM, were added in increasing amounts as indicated (given as fold excess over RNCs). Efficiency of membrane targeting was tested by treatment with proteinase K and is given as % protection. In lanes 1 and 7 undigested samples are shown (total). Lanes 2 and 8 present samples that were treated with proteinase K in the absence of microsomes (arrows indicate a fragment slightly smaller than ppl86 that is presumably protected from proteolysis by a cytosolic protein). The star marks the position of the 30 amino acid fragment protected by the ribosome; ppl86 indicates the position of the ppl86 chain.

To test for SRP-independent targeting we treated the isolated RNCs with *N*-ethylmaleimide (NEM) to inactivate SRP. It is known that membrane binding of ribosomes (Bacher et al., 1996) and the release of the nascent chain by puromycin (data not shown) are not affected by treatment with NEM. The function of NAC is also predicted to be insensitive to NEM-treatment because both subunits of NAC lack cysteines (Kanno et al., 1992; Yotov and St-Arnaud, 1996). As expected, the NEM-treated RNCs were still targeted to the membrane (Figure 15, lane 9). However, the efficiency of their membrane binding was much reduced in the presence of competing nontranslating ribosomes (Figure 15, lanes 10 to 12).

These results show that SRP-independent targeting in the presence of NAC and other cytosolic factors is not restricted to the wheat germ translation system. They provide further evidence for a model where in the absence of SRP, RNCs compete with nontranslating ribosomes for common binding sites but are given a competitive advantage over these ribosomes in the presence of SRP.

3.2.4. SRP-Independent Targeting to Reconstituted Proteoliposomes

To approach the mechanism by which SRP confers an advantage to RNCs in their competition with nontranslating ribosomes, we performed targeting assays with reconstituted proteoliposomes containing only the SRP receptor and the Sec61p complex purified from canine rough microsomes. RNCs carrying ppl86 were generated in a wheat germ extract and bound to Sec61p complex in proteoliposomes in the presence or absence of competing ribosomes. The targeting efficiency was assayed by proteinase K-treatment as discussed before.
As with native microsomes, in the absence of SRP, targeting was greatly reduced when competing nontranslating ribosomes were added (Figure 16, compare lanes 4 to 6 to lane 3). Binding of SRP allowed efficient targeting of RNCs even in the presence of competing ribosomes (Figure 16, lanes 7 to 10).

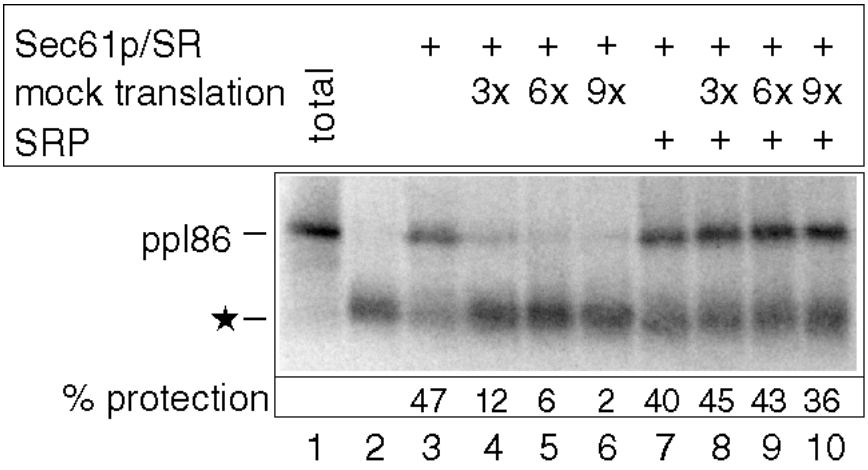

Figure 16: Targeting of ppl86 to reconstituted proteoliposomes. RNCs of ppl86 generated in a wheat germ system were incubated with reconstituted proteoliposomes containing purified SRP receptor (SR) and Sec61p complex (Sec61p) in the absence (lanes 3 to 6) or presence (lanes7 to 10) of SRP. Where indicated, mock translation mixture was added (given as fold excess over RNCs). Membrane targeting was tested using the protease protection assay. Lane 1 shows an undigested sample (total), all other samples were treated with proteinase K. ppl86 denotes the ppl86 chain. The star marks the position of the ribosome protected fragment of about 30 residues.

When proteoliposomes containing only the Sec61p complex were used in the targeting assay, the RNCs carrying ppl86 were targeted to the membrane independently of SRP. As before, upon addition of competing ribosomes SRP-independent targeting was inhibited (data not shown). In the presence of SRP, very little membrane binding of RNCs occurred (<5%, data not shown) and was completely abolished when competing ribosomes were added (data not shown). Presumably SRP remains bound to the signal sequence in the absence of SRP receptor and prevents insertion of the nascent chain into the translocation channel.
These results show that RNCs and nontranslating ribosomes compete for binding to the Sec61p complex. Furthermore, the binding of SRP to the signal sequence and to SRP receptor in conjunction with the interaction of RNC and Sec61p complex are necessary and sufficient to overcome the competition by nontranslating ribosomes.

3.2.5. Binding of the Signal Sequence to the Sec61p Complex Is Necessary for Gaining a Competitive Advantage

We then wanted to clarify the significance of the interaction between RNCs carrying a signal sequence and the Sec61p complex for the SRP-mediated competitive advantage. Thus, we asked whether binding of the signal sequence-bearing ribosome to the Sec61p complex is sufficient for gaining an SRP advantage or whether the signal sequence needs to be recognized by the Sec61p complex. To this end, we carried out competition experiments with a mutant ppl chain lacking 3 leucines within the hydrophobic core of the signal sequence (pplΔ13-15; Jungnickel and Rapoport, 1995). The mutant signal sequence binds to SRP, albeit with a 30% reduced efficiency compared to wild type preprolactin (judged by photocrosslinking; Jungnickel and Rapoport, 1995). However, the signal sequence is not bound by the Sec61p complex and therefore is non-functional for translocation. RNCs carrying the ppl86 fragment are targeted to the membrane but the transition from loose to tight ribosome binding to the Sec61p complex does not take place and the full length protein bearing the deletion is translocated across the ER membrane only with a low efficiency (0.5-2.5%; Jungnickel and Rapoport, 1995). When membrane-bound RNCs carrying ppl86Δ13-15 were subjected to treatment with proteinase K, both in the absence and presence of SRP, the majority of the nascent chains was protected (data not shown). However, a fraction of ppl86Δ13-15 was also degraded to a fragment of about 50 amino acids (data not shown). In the presence of competing nontranslating ribosomes, the 86 amino acid and the 50 amino acid fragments disappeared and the ribosome-protected 30 amino acid fragment became more prominent (data not shown). Ribosome competition was equally effective in samples with and without SRP (table 1, A). Thus, addition of SRP does not confer a competitive advantage to ppl86Δ13-15 even though the signal sequence binds SRP. Therefore, we conclude that a functional signal sequence is necessary for gaining an SRP-mediated competitive advantage over nontranslating ribosomes.

	RNC	competitor ribosomes	competition -SRP	competition +SRP
A	ppl86 Δ13-15 (s)	non-translating (s)	yes	yes
B	ppl59 (s)	non-translating (s)	yes	yes
C	ppl59 (2)	non-translating (1)	yes	yes
D	ppl59 (1)	non-translating (2)	no	no

Table 1: Binding of the signal sequence to the Sec61p complex is necessary for gaining a competitive advantage. (s) indicates samples where ribosomes and RNCs were added to the microsomes at the same time. (1) & (2) indicate the order in which ribosomes or RNCs were added to the microsomes.

In the ribosome competition assay, we also tested a shorter fragment of the wildtype preprolactin chain containing the 59 amino terminal residues (ppl59). RNCs carrying ppl59 represent an early translocation intermediate; although the signal sequence can bind to SRP (Jungnickel and Rapoport, 1995) it is too short to interact productively with the translocation channel. Therefore, the RNCs bind to the ER membrane only loosely and the transition to tight binding does not take place (Jungnickel and Rapoport, 1995). Since nascent chains of membrane-bound RNCs bearing the ppl59 chain are accessible to proteinase K, we used a sedimentation assay to measure RNC binding to microsomes. After incubation of the membranes with ribosomes and RNCs, the sample was loaded on a sucrose cushion containing physiological salt concentrations and the membranes were sedimented. The sedimentation of the microsomal membranes was verified with immunoblots using antibodies against Sec61α. The amount of membrane-bound ppl59 was determined by comparing the pellet and supernatant fractions. In samples lacking microsomes no ppl59 was recovered in the pellet fraction (data not shown). Competition experiments showed that RNCs carrying ppl59 cannot gain an advantage over competing nontranslating ribosomes even in the presence of SRP (table 1, B).

Furthermore, when the membrane binding sites were saturated with nontranslating ribosomes before addition of ppl59, the binding of the RNCs was prevented completely both in the absence and presence of SRP (table 1, C). However, when RNCs carrying ppl59 were bound to the microsomes first, they remained bound even when a large excess of nontranslating ribosomes was added (table 1, D).
These data indicate that, besides SRP binding, a successful competition with nontranslating ribosomes requires a productive interaction of the signal sequence with the Sec61p complex. Also, in the case of ribosomes or RNCs that can only bind loosely to the Sec61p complex, there is little exchange between the pools of free ribosomes and membrane-bound ribosomes. It seems that a ribosome or RNC once bound to Sec61p remains bound and will not be replaced by nontranslating ribosomes or by RNCs only capable of a loose interaction with Sec61p. The situation is different for RNCs carrying ppl86 which in the presence of SRP bind to microsomal membranes even when the membranes had been presaturated with nontranslating ribosomes (see Figure 12).

3.2.6. SRP Binding Gives a Competitive Advantage to Ribosomes Synthesizing Integral Membrane Proteins with A Signal Anchor

We wanted to verify that the SRP-mediated competitive advantage is granted not only to ribosomes synthesizing secretory proteins, but also to those synthesizing a membrane protein with a signal anchor. As a substrate we chose a short N-terminal fragment of the integral membrane protein leader peptidase (Figure 17A). Leader peptidase has two membrane anchors; the first membrane spanning domain serves as a signal anchor with the N-terminus located in the lumen of the ER. A cytosolic loop and a second membrane anchor follow, leaving the C-terminal portion of the protein in the ER lumen.

For competition experiments, ribosome-nascent chain complexes of a 57 residue N-terminal fragment of leader peptidase were generated in the wheat germ system (lep57, Figure 17A). Photocrosslinking verified binding of SRP to the signal anchor (data not shown). Membrane-bound RNCs carrying lep57 reflect the high salt

resistant stage of ribosome binding to the translocation channel; at this point the signal anchor is bound by the Sec61p complex (W. Mothes, personal communication).

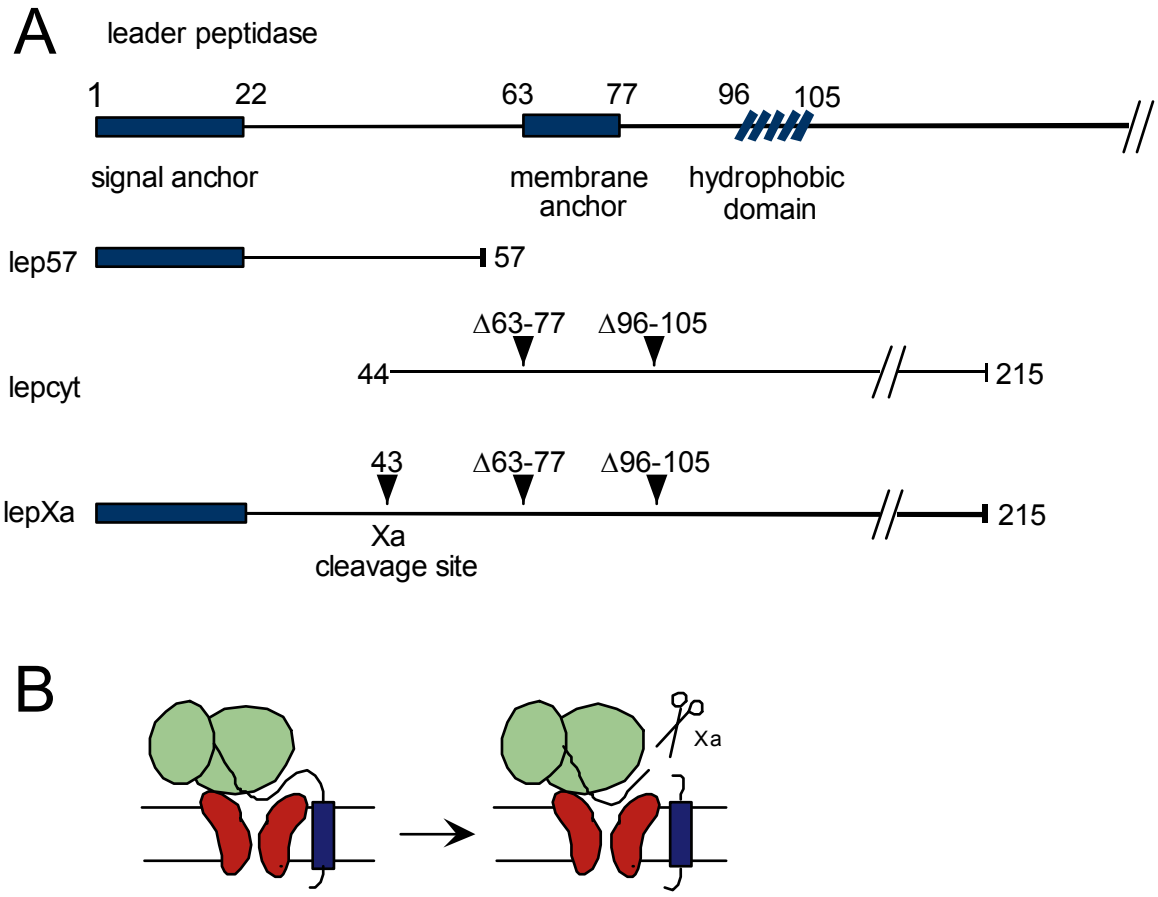

Figure 17: Fragments of leader peptidase were used to study membrane binding of different populations of RNCs. (A) The composition of wildtype leader peptidase is shown in comparison to other constructs used. lep57 represents a fragment containing only the signal anchor and adjacent residues. Lepcyt is a fragment starting at position 44 and lacking both the second membrane anchor and a short hydrophobic domain following the second anchor. lepXa contains the first membrane anchor but both the second membrane spanning domain and the following hydrophobic stretch are deleted. In addition, a
factor Xa cleavage site has been introduced at position 43. (B) Ribosomes synthesizing cytosolic domains of membrane proteins remain bound to the translocation site. Using the lepXa construct shown above, it has been shown that the translating ribosome remains bound to the translocation channel (Mothes et al., 1997) The cytosolic domain of lepXa is found in close proximity to the Sec61p complex even after the preceding membrane anchor has contacted the lipid phase of the ER membrane (left). This is the case even when the physical connection between membrane spanning domain and cytosolic domain has been severed by treatment with factor Xa (right).

We used the sedimentation assay to monitor membrane targeting of RNCs carrying lep57 in the absence and presence of competing nontranslating ribosomes. As for the secretory protein preprolactin in the absence of SRP, nontranslating ribosomes were competing with lep57 for membrane binding. Yet, when its signal anchor was bound by SRP, lep57 gained a competitive advantage over nontranslating ribosomes (table

2, A). Thus, we conclude that SRP binding gives a competitive advantage to RNCs carrying either a signal sequence or a signal anchor.

3.2.7. Membrane Binding of Ribosomes Synthesizing Cytosolic Proteins

We also wanted to compare membrane binding of nontranslating ribosomes and of ribosomes translating proteins destined to remain in the cytosol. As an example for a cytosolic protein, we created a nascent chain of leader peptidase lacking all hydrophobic regions. It included amino acids 44 to 215; both the second membrane anchor and the following short hydrophobic stretch were deleted, leaving a total of 149 amino acids (Figure 17A, lepcyt). First, we used ribosomes carrying the leader peptidase fragment for competition of membrane binding of RNCs bearing the ppl86 chain. To this end, a sedimentation assay was performed using ppl86 and lepcyt chains that had been radiolabeled with ^{35}S-methionine. Since the two populations of nascent chains were separated by SDS-PAGE, we were able to monitor their membrane binding independently. When ppl86 and lepcyt RNCs were added to the microsomes simultaneously in the absence of SRP, membrane binding of RNCs carrying the ppl86 chain was reduced with addition of increasing amounts of RNCs carrying the lepcyt polypeptide (table 2, B). Therefore we conclude that in the absence of SRP, RNCs synthesizing cytosolic proteins can compete with RNCs carrying a signal sequence for binding to the ER membrane. However, in the presence of SRP, ribosomes translating ppl86 were efficiently bound to the microsomes even when a large excess of RNCs carrying lepcyt was present (table 2, B). This shows that SRP binding gives RNCs containing a signal sequence a competitive advantage in membrane targeting over ribosomes synthesizing cytosolic proteins.
After RNCs carrying ppl86 had been prebound to the membrane, even a large excess of lepcyt RNCs did not replace them from the Sec61p complex (table 2, C). Conversely, when ppl86 RNCs were added to microsomes where all binding sites had been saturated with ribosomes carrying the lepcyt fragment, no membrane association of ppl86 was detected without SRP. However, efficient targeting occurred in the presence of SRP (table 2, D).

We also used RNCs carrying lepcyt in targeting assays with competing nontranslating ribosomes. Both populations of ribosomes competed for binding sites at the ER membrane (table 2, E). However, when microsomes had been saturated with one kind of ribosomes, these were not replaced even after addition of a large excess of the other kind of ribosomes (table 2, F and G). Next, we used an excess of ppl86 RNCs to compete ribosomes translating lepcyt for binding to microsomes. For ppl86, efficient targeting took place whenever SRP was present (table 2, H, I and J). Even when RNCs carrying lepcyt had been prebound to the membrane, ppl86 RNCs with bound SRP were able to displace them (table 2, I)

	RNC	competitor ribosomes	competition -SRP	competition +SRP
A	lep57 (s)	non-translating (s)	yes	no
B	ppl86 wildtype (s)	leader peptidase cytosolic (s)	yes	no
C	ppl86 wildtype (1)	leader peptidase cytosolic (2)	no	no
D	ppl86 wildtype (2)	leader peptidase cytosolic (1)	yes	no
E	leader peptidase cytosolic (s)	non-translating (s)	yes	ND
F	leader peptidase cytosolic (1)	non-translating (2)	no	ND
G	leader peptidase cytosolic (2)	non-translating (1)	yes	ND
H	leader peptidase cytosolic (s)	ppl86 wildtype (s)	yes	yes
I	leader peptidase cytosolic (1)	ppl86 wildtype (2)	no	yes
J	leader peptidase cytosolic (2)	ppl86 wildtype (1)	yes	yes

Table 2: Competition experiments using RNCs carrying different fragments of leader peptidase. (s) indicates samples where ribosomes and RNCs were added to the microsomes at the same time. (1) & (2) indicate the order in which ribosomes or RNCs were added to the microsomes.

To summarize, ribosomes synthesizing cytosolic proteins as well as nontranslating ribosomes compete for common binding sites with ribosomes translating secretory nascent chains or membrane proteins. Binding of SRP confers a competitive advantage to RNCs that can interact tightly with the Sec61p complex.

In fact, these RNCs can secure a translocation site even when the ER membrane is saturated with loosely bound ribosomes.

3.2.8. Ribosome Binding During Integration of Multispanning Membrane Proteins

For integration of multispanning membrane proteins it has been suggested that the ribosome experiences repeated targeting cycles after translation of cytosolic domains. However, recent data indicate that the ribosome remains bound to the Sec61p complex while it is translating a cytosolic domain.

These data were derived from experiments with translation intermediates of leader peptidase containing only the signal anchor and the adjacent cytosolic domain. To allow detachment of the cytosolic domain from the signal anchor, a consensus site for cleavage by factor Xa was introduced at position 43 (Figure 17A, lepXa). After digestion with factor Xa, the ribosome-associated fragment of lepXa is identical to lepcyt, the cytosolic fragment of leader peptidase that has been used before (compare lepcyt and lepXa in Figure 17A).

Photocrosslinking experiments with membrane-bound ribosomes carrying the lepXa fragment have shown that the cytosolic portion of lepXa remains in close proximity to the Sec61p channel even after cleavage of the nascent chain by factor Xa (Mothes et al., 1997; see also Figure 17B). Under these conditions, even though there is no physical connection between cytosolic domain and membrane anchor, the ribosomes stay tightly bound to Sec61p (Mothes et al., 1997).

We were interested in comparing the membrane binding of RNCs carrying the Xa-cleaved lepXa to binding of ribosomes carrying the equivalent lepcyt chain. To this end, we performed competition assays with nontranslating ribosomes and RNCs carrying ppl86.

First, ribosomes bearing the lepXa fragment were targeted to microsomes in the presence of all cytosolic factors but SRP. Then, the sample was treated with factor Xa, resulting in cleavage of about 50% of the lepXa chains. Next, competitor ribosomes were added, either nontranslating ribosomes or RNCs carrying ppl86 with or without bound SRP. The efficiency of membrane binding of the RNCs was tested with the sedimentation assay. Both the uncleaved and the cleaved nascent chains as

well as the ppl86 chain were detected when the sample was separated in SDS-PAGE, allowing for independent monitoring of all populations of RNCs.

	RNC	competitor ribosomes	competition -SRP	competition +SRP
A	lepXa (1)	non-translating (2)	no	no
B	lepXa (1)	ppl86 (2)	no	no

Table 3: Competition experiments using membrane-bound ribosomes synthesizing cytosolic domains of integral membrane proteins. (1)/(2) indicates that before addition of competing ribosomes, RNCs carrying lepXa were bound to the ER membrane and digested with factor Xa.

As expected, nontranslating ribosomes and RNCs carrying ppl86 without SRP did not compete off the Xa cleaved or the uncleaved lepXa RNCs (table 3, A and B). However, to our surprise, RNCs carrying ppl86 were not able to displace ribosomes carrying the lepXa fragments, even in the presence of SRP (table 3, B). The amount of cleaved and uncleaved lepXa found in the membrane fraction was not reduced even when a 20fold excess of ppl86 RNCs was added, although ribosomes carrying the lepcyt chain were displaced by RNCs carrying ppl86 with bound SRP (see table 2). Nevertheless, we did see a small increase of ppl86 bound to microsomes in the presence of SRP, probably accounting for competition of these RNCs with nontranslating ribosomes present in the sample. This observation confirms that functional SRP was indeed bound to ppl86.

These results suggest that binding of ribosomes translating a cytosolic domain of a multispanning membrane protein is distinct from binding of ribosomes synthesizing cytosolic proteins. Our data indicate that a ribosome translating a multispanning membrane protein will not leave the translocation site before integration of the membrane protein is completed.

3.3. Structural Analysis of Ribosome Binding to the ER Membrane

Two different stages of ribosome binding to the translocation channel in the ER membrane have been described (Wolin and Walter, 1993; Crowley et al., 1994; Jungnickel and Rapoport, 1995). Nontranslating ribosomes, ribosomes synthesizing cytosolic proteins and ribosomes carrying short secretory nascent chains bind only loosely to Sec61p. The interaction is sensitive to high salt concentrations. A transition to tighter binding takes place for ribosomes synthesizing secretory proteins upon elongation of the nascent chain and binding of the signal sequence to the Sec61p complex. This tighter interaction is characterized by resistance to high salt concentrations.
Biochemical data (Crowley et al., 1994) indicate that in the tight binding state the ribosome-channel interaction might become much more intimate and that a continuous sealing exists around the ribosome-membrane junction. Using single particle cryo electron microscopy, we wanted to compare loose and tight ribosome binding to the translocon to determine whether any differences between these stages can be detected on a structural basis. Furthermore, we were interested in a comparison of the structural features of the purified Sec61p channel and the native translocation channel, which has been shown to contain other integral membrane proteins in addition to the Sec61p complex (Görlich et al., 1992a,b).

3.3.1. Preparation of Ribosome-Translocation Channel Complexes

We established a method for isolating stable complexes of ribosomes bound to translocation channels in detergent solution. These complexes contained either nontranslating ribosomes or RNCs carrying ppl86, representing loose or tight binding modes, respectively. For determination of a 3D structure by single particle cryo electron microscopy it is essential to generate a homogenous population of particles. Hence, we optimized the isolation procedure to yield a maximum of ribosomes with an attached channel and to minimize loss of nascent chains by hydrolysis of the peptidyl-tRNA.

First, ribosome-nascent chain complexes of ppl86 were assembled in the rabbit reticulocyte lysate system in the presence of reconstituted proteoliposomes containing purified Sec61p and SRP receptor (Figure 18A, lane 1). It has been shown before that the 86mer of ppl induces a high-salt resistant interaction of ribosome and translocation channel (Jungnickel and Rapoport, 1995). By comparing the total number of ribosomes and the number of nascent chains present in the *in vitro* translation reaction, we estimated that about 10-30% of the ribosomes in the translation reaction carried a nascent chain (data not shown). After binding of the RNCs, the proteoliposomes were floated in a sucrose step gradient under high salt conditions. Membrane- associated RNCs were recovered in the floated fraction (Figure 18A, lane 5) whereas unbound RNCs and ribosomes bound only loosely to Sec61p remained in the bottom part of the sucrose gradient (data not shown). The interaction of ribosomes and membranes was mediated by the translocation channel since very little ppl86 was found in the floated fraction when vesicles lacking Sec61p and SRP receptor were used (data not shown). Taken together, these data indicate that after flotation only ribosomes tightly bound to the Sec61p complex are recovered.

Following flotation, the membranes were solubilized in a buffer containing 1.5% digitonin. Under these conditions, the Sec61p complex remains ribosome-associated whereas SRP receptor is soluble. The ribosome-channel complexes were then pelleted to separate them from an excess of unbound membrane proteins (Figure 18A, lane 9). Most of the 86mer remained bound to ribosomes as peptidyl-tRNA, as demonstrated by precipitation with cetyltrimethylammonium bromide (CTABr; Figure 18A, lane 10 vs. lane 9). In fact, the complex of ribosome, 86mer and Sec61p channel proved to be very stable, even when generated with native membranes instead of proteoliposomes (Figure 18B). Judged by CTABr precipitation, the nascent chain stayed ribosome-associated for at least 5 hours (Figure 18B, lane 14 vs. lane 13). In addition, the nascent chain remained protease-resistant, indicating that it is located inside the ribosome-channel complex (Figure 18B, lane 15 vs. lane 13).
These data demonstrate that stable ribosome-channel complexes representing the tight binding stage can be isolated.

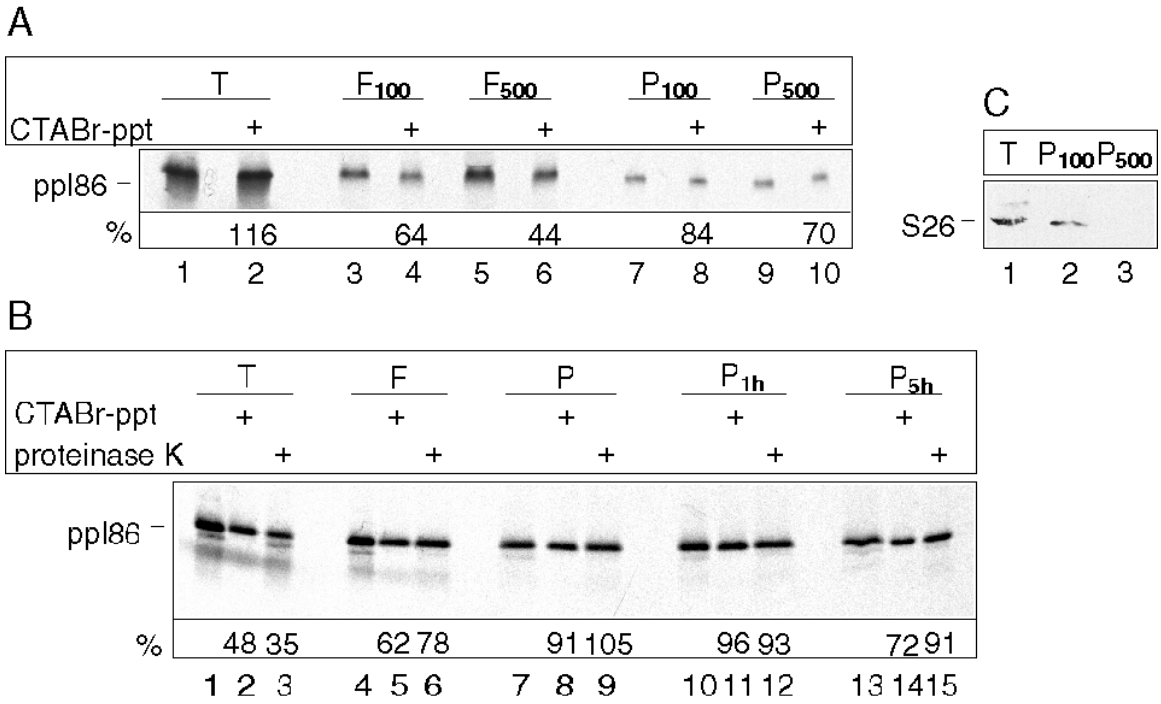

Figure 18: Preparation of ribosome-translocation channel complexes. (A) RNCs of ppl86 were assembled in reticulocyte lysate in the presence of proteoliposomes containing purified Sec61p complex and SRP receptor. The vesicles were subjected to flotation in a sucrose step gradient at either low (100mM potassium acetate) or high (500mM potassium acetate) salt concentrations. Membranes with bound RNCs were solubilized in digitonin at the indicated salt concentration and the ribosomes were isolated by centrifugation. Equivalent aliquots of the original translation (T), after flotation (F_{100} and F_{500}) or after sedimentation (P_{100} and P_{500}) were analyzed by SDS-PAGE. Each sample was also precipitated with CTABr (CTABr-ppt) and the amount of precipitable material is given below the lanes (%). (B) As in (A), except that PKRM were used, and flotation and solubilization were performed at 500mM potassium acetate. Aliquots of all samples were analyzed by CTABr precipitation (CTABr-ppt, lanes 2, 5, 8, 11 and 14) and in a protease-protection assay (proteinase K, lanes 3, 6, 9, 12 and 15). In addition, the final pellet fraction was incubated on ice for either 1h or 5h to test the stability of the ribosome-channel complex (P_{1h}, P_{5h}). Four times more material was loaded in the F and P samples than in T samples. (C) As in (A), except that a non-programmed translation mix was used. The ribosomes in the original sample (T, 10% loaded) and in the final pellet fractions (P_{100} and P_{500}) were detected by immunoblotting using an antibody raised against the ribosomal protein S26. ppl86 points to the position of the nascent chain.

To prepare complexes of ribosomes loosely bound to the translocation channel we used unprogrammed reticulocyte lysate as a source of nontranslating ribosomes. Flotation and solubilization were carried out under low salt conditions. Immunoblotting with an antibody directed against the ribosomal protein S26 demonstrated that nontranslating ribosomes were present in the final fraction only at low salt concentration (Figure 18C, compare lanes 3 and 2). Again, this provided evidence that only ribosomes tightly associated to Sec61p are recovered under high salt conditions.

Taken together, these data showed that we generated stable ribosome-translocation channel complexes with or without a defined nascent chain. Isolation of the

complexes allowed a structural analysis of the loose and tight stage of ribosome binding to the Sec61p complex.

3.3.2. Structures of Ribosomes Bound to Purified Sec61p Complex

Previously, a 3D structure of nontranslating yeast ribosomes bound to purified yeast Sec61p complex has been published (Beckmann et al., 1997). This structure, representing ribosomes bound only loosely to the Sec61p complex, revealed a sizable gap between ribosome and channel with just one connection bridging the ribosome-channel junction. To test whether the ribosome-Sec61p channel junction becomes more intimate after binding of a signal sequence to the Sec61p complex, we compared 3D maps of ribosomes bound to the channel formed by the purified Sec61p complex in the absence and presence of a nascent chain.
Samples for electron microscopy were prepared as described above except that ppl86 was translated without radioactive methionine. After sedimentation, the ribosome-channel complexes were resuspended, frozen and analyzed by cryo electron microscopy. Ribosomes were readily identifiable on the grid. In addition, some ring-like Sec61p complexes (Hanein et al., 1996) were visible in the background. Using a previously determined 3D structure of the rabbit ribosome as a first reference (Morgan et al., 2000) we determined 3D maps of the isolated ribosome-channel complexes. Since the electron density is continuous, an appropriate threshold level must be chosen to represent and interpret the structure. The density above this threshold should correspond to the volume of the ribosome-channel complex (see Materials and Methods). We have defined a ribosomal volume of 100% as the threshold that encloses the calculated volume of a rabbit ribosome and, in addition, allows expected features of the ribosome to be recognized. Due to some ambiguity in the volume calculation, the appropriate threshold may deviate from this 100% value. Moreover, if some of the ribosomes used for the structural analysis were lacking channels, a lower threshold may be appropriate to visualize the full volume of the channel.

To our surprise, a comparison of the structures of ribosome-Sec61p channel complexes in the absence (Figure 19, top row) or presence (Figure 19, bottom row)

of the ppl86 chain revealed no differences at a 25Å resolution (see Figure 19A, C, E and G for frontal views of the ribosome-channel complexes, and Figure 19B, D, F and H for views from the ER lumen; compare also table 4). Furthermore, the 3D maps are very similar to the previously described structure of the yeast complex containing nontranslating ribosomes and purified Sec61p complex (Beckmann et a., 1997; and our unpublished results).

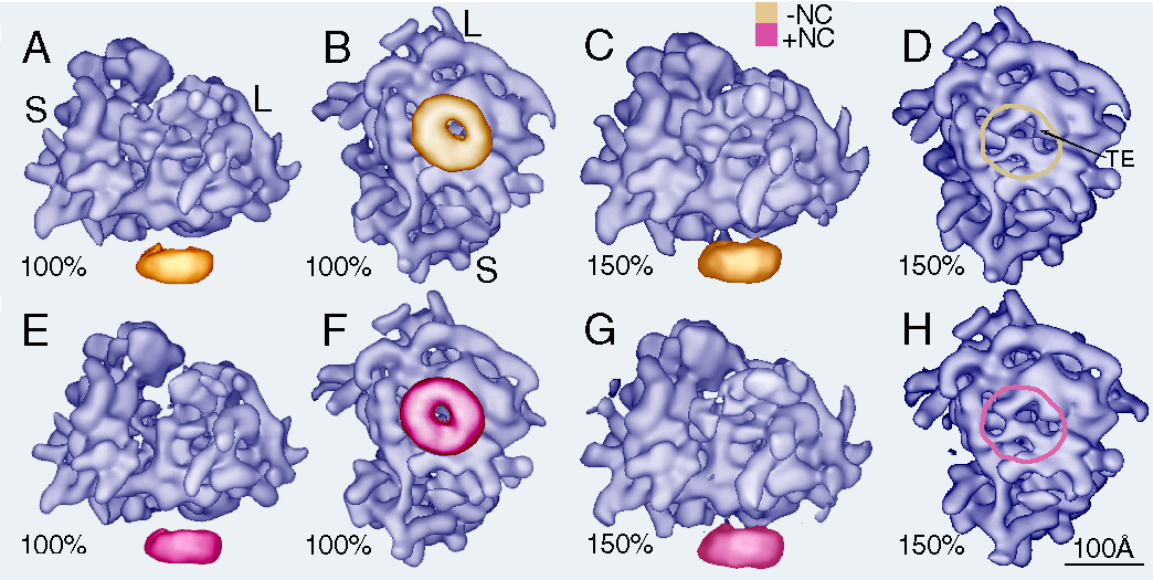

Figure 19: Ribosomes and ribosome-nascent chain complexes associated with purified Sec61p complexes. (A) A 3D map of ribosome-Sec61p channel complexes lacking a nascent chain is viewed along the plane of the ER membrane (frontal view). The threshold level was chosen to encompass 100% of the ribosomal volume. The small (S) and large (L) ribosomal subunits are indicated. (B) As in (A), but viewed from the ER lumen (bottom view). This view is generated by a 90° rotation about the horizontal axis, followed by a 90° rotation in the image plane. (C) As in (A), except that the threshold level was lowered to encompass 150% of the expected ribosomal volume. (D) As in (C), but shown in bottom view. The Sec61p channel is shown as a circumference (outlined in gold) to reveal the ribosomal nascent chain tunnel exit (TE). (E) A 3D map of ribosome-Sec61p channel complexes carrying the ppl86 chain is shown in front view with the threshold level set to enclose 100% of the ribosomal volume. (F) Same as (E), but shown in bottom view. (G) Same as (E), except with a threshold that encompasses 150% of the expected ribosomal volume. (H) As in (G), but shown in bottom view. The Sec61p channel with an inserted nascent chain is shown as a circumference (outlined in red) to reveal the ribosomal nascent chain tunnel exit.
The color code for the Sec61p complexes containing or lacking the ppl86 chain (-/+NC) is given as a vertical bar. Scale bar=100Å.

At a 100% threshold level, the ring formed by the Sec61p channel had an outer diameter of about 85Å and included a central pore (Figure 19B and F). The pore was aligned with the nascent chain exit site of the large ribosomal subunit to provide a continuous passage from the ribosomal peptidyltransferase center into the lumen of the endoplasmic reticulum (see Figure 19D and H, the golden and pink channel outlines surround the tunnel exit sites, indicated by TE). Between the ribosome and

the Sec61p channel no connection was seen at the threshold level chosen here. Instead, as described before for the yeast ribosome-channel complex (Beckmann et al., 1997; and our unpublished results), a substantial gap of about 20Å width was present (Figure 19A and E). The gap became narrower and some connections appeared when the threshold level was lowered to enclose 150% of the calculated ribosomal volume (Figure 19C and G). However, even in the presence of a nascent polypeptide chain engaged in translocation, the ribosome-channel junction does not become more intimate and there were no significant differences detectable between ribosome-Sec61p complexes containing and lacking a nascent chain.

Table 4: Summary of datasets

ribosomes	membranes	nascent chains	particles	resolution*
rabbit	proteoliposomes	none	9942	25Å
rabbit	proteoliposomes	ppl86	7902	25Å
rabbit	PKRM	none	6914	27Å
rabbit	PKRM	ppl86	6863	29Å
canine	KRM	mixed	6488	27Å

*Resolution was determined with the $FSC_{0.5}$ criterion. For each dataset, pairs of 3D volumes were calculated with increasing numbers of particles up to the total number divided by 2. The appropriate volumes were then compared and their resolution plotted as a function of increasing particle number.
This allowed us to estimate the resolution of the final complete 3D dataset by extrapolation.

3.3.3. Ribosomes Bound to Native Translocation Channels

In addition to Sec61p, in native membranes other components of the translocation machinery have been found to be tightly associated with ribosomes (Görlich et al., 1992a). These membrane proteins might be part of the translocation channel. We therefore used PKRM to pursue a structural analysis of complexes consisting of ribosomes bound to native translocation channels.
Ribosome-channel complexes with or without ppl86 chains were prepared and subjected to cryo electron microscopy as described above except that PKRM were added instead of proteoliposomes containing purified membrane proteins. To remove

ER membrane proteins that are only loosely attached to the ribosome-channel complexes, the membranes were always solubilized under high salt conditions.

The resulting 3D structures had a resolution of 27Å for ribosome-channel complexes without a nascent chain and 29Å for complexes with a ppl86 chain (table 4). These 3D maps showed essentially the same features as seen previously in structures of ribosomes bound to purified Sec61p channel (Figure 20A, C, D and F, front view; Figure 20B and E, bottom view). As before, there were only small differences between ribosome-channel complexes with or without nascent chains. At a 100% threshold level (Figure 20A, B, D and E) no connection was visible between ribosome and translocation channel. Yet, the central pore of the channel was precisely aligned with the nascent chain exit site of the large ribosomal subunit. Again, a 20Å gap was present between ribosome and channel. When the threshold level was set to 150% of the ribosome volume, the gap became narrower and a few connections appeared (Figure 20C and F).
Interestingly, in contrast to the channel formed by the purified Sec61p complex, an additional domain was visible on the lumenal side of the native translocation channel. This domain was precisely oriented with respect to the ribosome and the channel (to be discussed in more detail later).

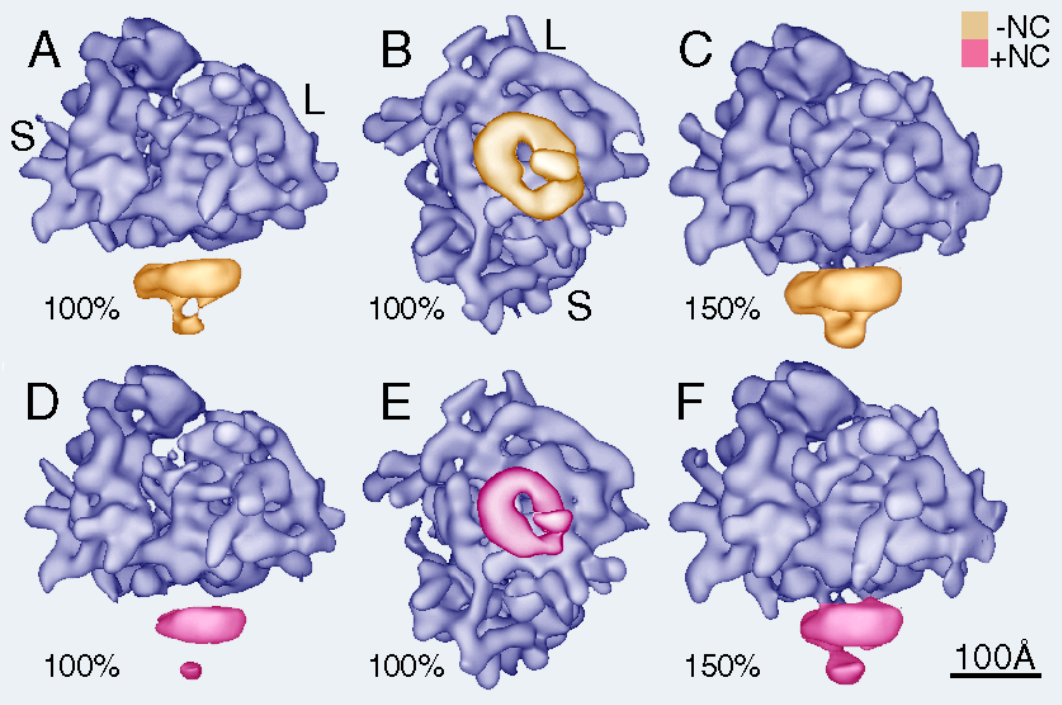

Figure 20: Ribosomes associated with the Sec61p complex derived from native membranes. (A) A 3D map of ribosomes bound to Sec61p complexes derived from PKRM is viewed along the plane of the membrane (frontal view). The threshold level was chosen to encompass 100% of the expected ribosomal volume. The small (S) and large (L) ribosomal subunits are indicated. (B) As in (A), but viewed from the ER lumen (bottom view). (C) As in (A), but with a threshold that encompassed 150% of the expected ribosomal volume. (D) The ribosome-nascent chain complex carrying ppl86 bound to Sec61p channel derived from PKRM is shown in front view with a threshold level that encompasses 100% of the ribosomal volume. (E) As in (D), but shown in bottom view. (F) As in (D), except that the threshold level was chosen to encompass 150% of the expected ribosomal volume. The color code of the ribosomes containing or lacking nascent ppl86 chains (-/+NC) is given as a vertical bar. Scale bar=100Å.

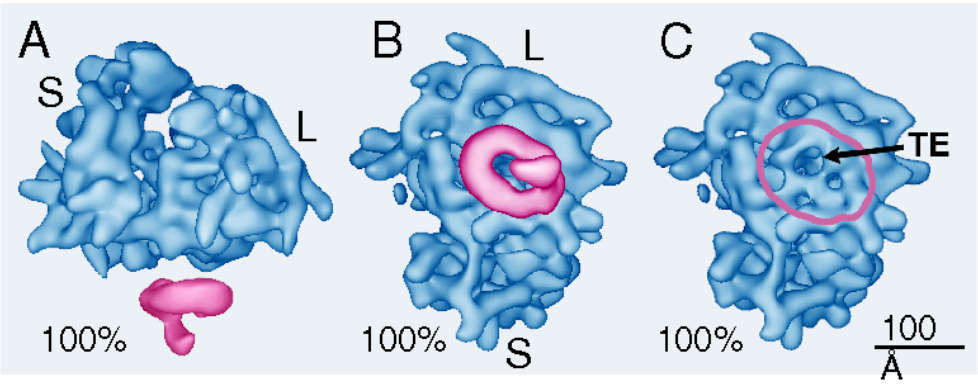

Figure 21: Ribosome-translocation channel complexes containing a heterogeneous mixture of endogenous nascent chains. (A) A 3D map of the complex of canine ribosomes and canine translocation channels derived from native high salt washed microsomes (KRM), with endogenous nascent chains present is shown in frontal view. The threshold level was set to enclose 100% of the ribosomal volume. (B) As in (A), but shown in bottom view. (C) As in (B), with the translocation channel shown as a circumference (outlined in red) to reveal the ribosomal tunnel exit (TE). Scale bar=100Å.

3.3.4. Ribosome-Channel Complexes with a Mixed Population of Nascent Chains

Using ppl86 as a model for a nascent chain that is inserted into the translocation channel, we have not been able to detect any changes in the ribosome-channel junction during the transition from loose to tight ribosome binding to the Sec61p channel. To exclude the possibility that our results were biased by the choice of ppl86 as the nascent chain we wished to examine ribosome-channel complexes representing different stages of translocation of a wide range of substrates. We therefore prepared ribosome-channel complexes derived from salt-washed microsomes (KRM). Ribosomes bound to these native ER membranes carry a mixture of endogenous nascent chains engaged in translocation, presumably nascent chains of secretory and membrane proteins at later stages of translocation. By using ribosome-channel complexes prepared from KRM we also compared endogenously formed channels to translocation complexes assembled *in vitro*.

After treating canine rough microsomes with high salt concentrations to remove ribosomes without nascent chains, these KRM were solubilized in digitonin under high salt conditions. Then, the ribosome-channel complexes were pelleted and analyzed by cryo electron microscopy as before.

A 3D map of canine ribosome-nascent chain complexes bound to canine Sec61p channel was generated at 27Å resolution (table 4). The canine ribosome proved to be very similar to the rabbit ribosome. Also, all features described previously for structures of ribosome-channel complexes were visible in the map derived from KRM (Figure 21A to C). The ribosomal exit site was found aligned with the central pore of the Sec61p channel (Figure 21C, the pink line shows the contour of the channel, TE marks the tunnel exit). As before in structures derived from PKRM, the prominent lumenal domain was visible (Figure 21A and B). Even with complexes containing a mixed population of nascent chains, no connections were seen between ribosome and channel and a gap of about 20Å was present at a threshold level of 100% (Figure 21A).

3.3.5. The Ribosome-Channel Junction

Data derived from biochemical studies have indicated that the ribosome-membrane junction becomes much more intimate when a nascent chain is inserted into the translocation channel (Crowley et al., 1994). However, our structural data show that at least at the resolution presented here, no significant differences are seen between ribosome-channel complexes lacking nascent chains and those actively engaged in translocation. Therefore, we wished to study the ribosome-channel junction in more detail. To do so, we chose the structure of ribosome-channel complexes derived from KRM as an example.

As for all structures described here, at a 100% threshold level, no connection was visible between ribosome and Sec61p channel (Figure 21A). Instead, a sizable gap of about 20Å was present. When the threshold level was lowered to include 150% of the calculated ribosome volume, the gap narrowed and a few connections between channel and large ribosomal subunit appeared (Figure 22A). At a threshold level set to 200%, the links connecting ribosome and channel became more prominent (Figure 22B). However, the ribosome-channel interface was not completely filled with electron-dense material even though at 200% the threshold level is so low that some background noise started to appear (shown in gray). In Figure 23A, a slice through the same ribosome-channel complexes is shown, contoured to represent threshold levels set to 100% (green), 150% (yellow) and 200% (white). The plane of the membrane (M) is indicated by white lines, PTC indicates the position of the peptidyltransferase center in the ribosome. Even at a contour level of 200%, a continuous passage from the ribosomal nascent chain tunnel into the cytosol was revealed (Figure 23A, dotted line). Clearly, a 200% threshold level is too low, since both the channel pore and an uninterrupted ribosomal tunnel from the PTC to the nascent chain exit site are no more visible (Figure 23A, white contour line). Since the gap is still visible at this unreasonably low threshold level, we conclude that it is a genuine feature of the ribosome-channel junction.

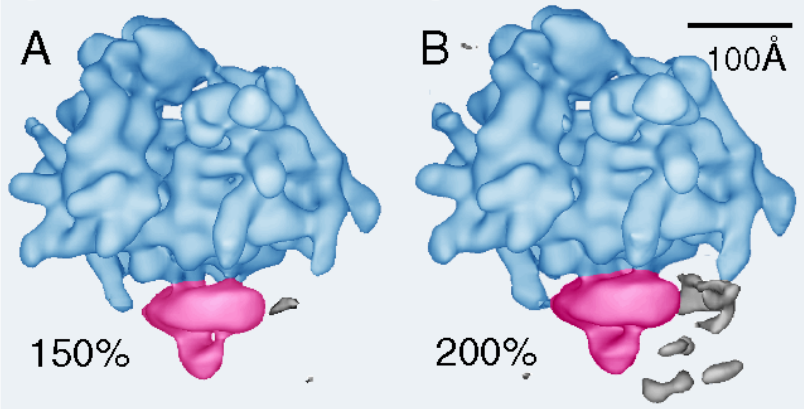

Figure 22: The gap between ribosome and translocation channel is present even at lower threshold levels. (A) A 3D map of ribosome-channel complexes derived from KRM shown in front view at a threshold level that encompasses 150% of the expected ribosomal volume. Gray features indicate background noise. (B) As in (A), except that the threshold level was set to enclose 200% of the expected ribosomal volume. Scale bar=100Å.

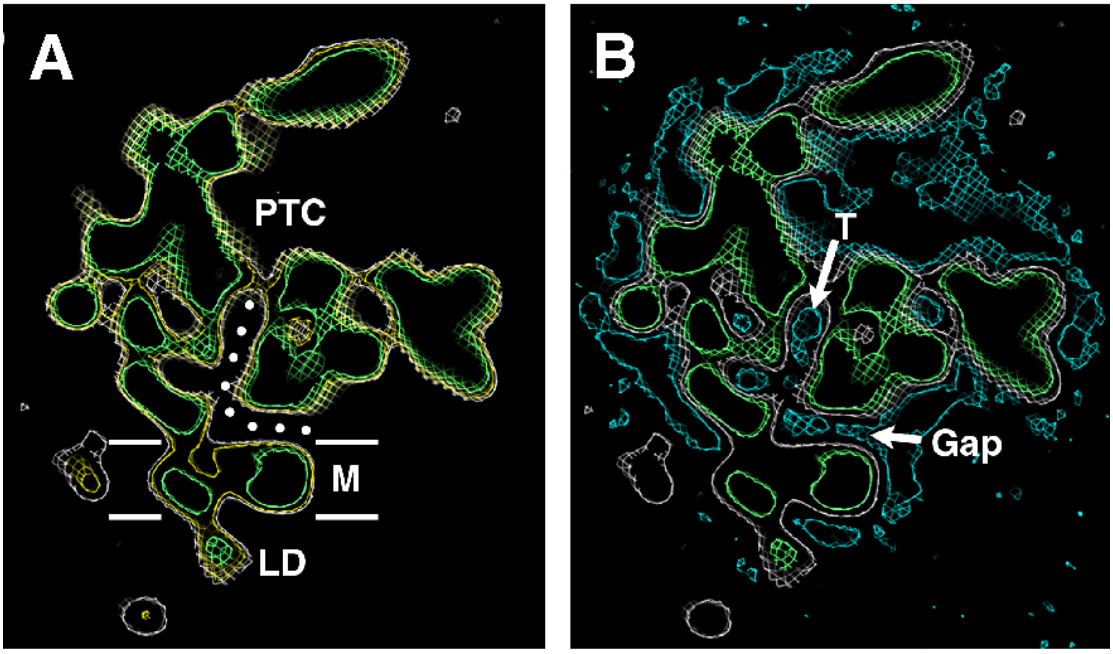

Figure 23: The gap is a genuine feature of ribosome-channel complexes. (A) A section through the map of ribosome-channel complexes derived from KRM. The channel is oriented at the bottom and the approximate position of the membrane is indicated (white lines, M). The map is contoured at 3 threshold levels, corresponding to 100% (green), 150% (yellow) and 200% (white) of the expected ribosomal volume. The gap and the ribosomal nascent chain exit tunnel form a continuous path (white dots) from the peptidyltransferase center (PTC) to the cytosol, with a diameter of about 20Å at the 200% threshold. The position of the lumenal domain is indicated (LD). Maps were displayed in "O" (Jones et al., 1991). (B) A view similar to that in (C), with two threshold levels for the ribosome-channel complex, 100% (green) and 200% (white). Superimposed on this view is a statistical map (in blue), which indicates regions that are lacking electron density with a confidence level greater that 99%.

A statistical analysis further strengthened this conclusion. We identified regions that contained neither protein nor RNA (Figure 23B; the blue areas are significant at a confidence level greater than 99%, T indicates the tunnel within the ribosome). These data show that the peptidyltransferase center, the central pore of the translocation channel and the gap between ribosome and channel are equally empty. A similar analysis performed with all other 3D structures confirmed that the gap is a significant feature of the ribosome-membrane junction.

We then compared the position of links between the large ribosomal subunit and the Sec61p channel in all structures presented here and in a similar structure of yeast nontranslating ribosomes bound to yeast Sec61p complex.
In Figure 24, an overlay is shown of slices through ribosome-channel complexes at the level of the ribosome-channel junction. The complex derived from S. cerevisiae is indicated by the dark green contour line of the ribosome. Mammalian ribosome-channel complexes containing either purified Sec61p channel or native channels, both in the absence and presence of a nascent chain are represented by all other contour lines. The light green channel outline represents the channel map derived from PKRM without nascent chains. The slices were aligned using the ribosomal tunnel exit as a reference (indicated by the arrow).
Three connections (dark shaded areas inside the channel contour) were identified as present in all of the structures, another connection (lighter shaded area above the tunnel exit) was present in most of the structures. Since the slices are superimposed onto each other, the darkest areas represent mass found most frequently in one location, whereas lighter areas indicate electron dense mass found only in some maps.

The conserved links between ribosome and channel are arranged around the central pore of the channel in a horseshoe shape. In structures derived from native ER membranes, the opening of the horseshoe towards the cytosol points away from the lumenal domain. There were never any connections visible on the side of the channel where the lumenal domain emerges nor in any position to completely seal the ring around the central pore (compare to Figure 22) indicating that the location of the gap in the ribosome-channel junction is conserved among all structures analyzed.

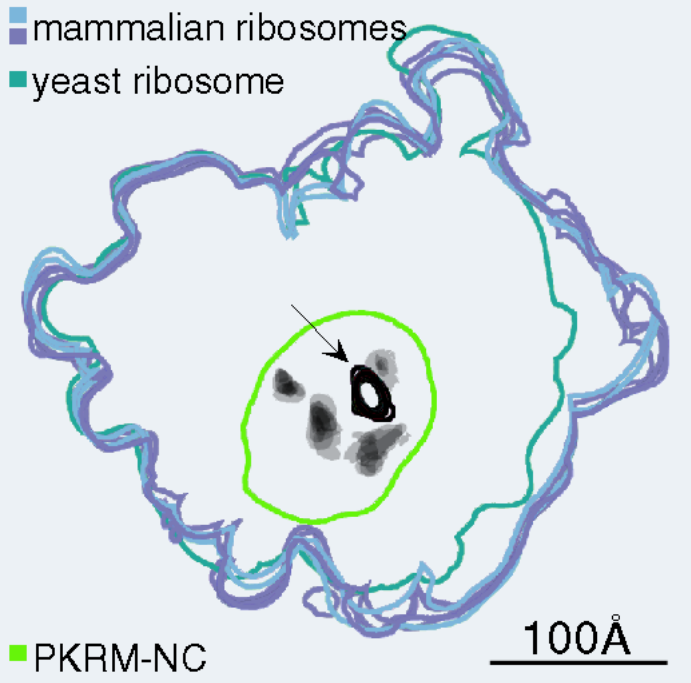

Figure 24: A discrete number of connections between ribosome and translocation channel are present in conserved positions. An overlay is shown of sections through ribosome-channel complexes in the plane of the ribosome-channel junction. The ribosome-channel complexes contained either nontranslating yeast ribosomes bound to Sec61p complex purified from S. cerevisiae (contoured in dark green) or mammalian ribosomes bound to mammalian translocation channels derived from native membranes or from reconstituted proteoliposomes containing purified Sec61p complex (all other contours). The maps are aligned with respect to the ribosomal nascent chain tunnel exit (indicated by the arrow). The outline of the translocation channel derived from PKRM in the absence of a nascent chain is given as a reference (light green outline). Scale bar=100%.

We then raised the threshold level for contouring of the ribosome-channel complexes to include only high-density structures representing the ribosomal RNA rather than ribosomal proteins. In these maps distinct features of the ribosome were found to be located at positions were the conserved links to the channel had been seen (data not shown) suggesting that specific regions of ribosomal RNA take part in establishing the connection to the translocation channel.

Taken together, these data show that the ribosome is bound to the Sec61p channel by a discrete number of links, presumably connected to or even formed by distinct regions of ribosomal 28S RNA. These links are precisely located with respect to both the ribosome and the translocation channel. In addition, our results indicate that even when a nascent chain is being transferred through the channel, the ribosome-membrane junction is not completely sealed towards the cytosol.

3.3.6. Comparison of Purified Sec61p Channel and Native Translocation Channel

While our data show that the ribosome-channel junction is similar for structures derived from the purified Sec61p complex or from native membranes, the channels themselves differ significantly. In Figure 25, the purified Sec61p channel without a nascent chain (Figure 25A, left panel, and Figure 25B) and the native translocation channel (derived from KRM, Figure 25A, middle panel, and Figure 25C) are depicted at a threshold level of 110%. The channels are shown without the ribosomes either as viewed from the lumen of the ER (Figure 25A, top row), as viewed from the ribosome (Figure 25B and C, left panel) or in frontal view (Figure 25A, bottom row, and Figure 25B and C, middle and right panel).

Compared to the purified Sec61p channel, the native channel contained an additional lumenal domain (Figure 25A, middle vs. left panel) that was precisely oriented with respect to both ribosome and membrane-spanning regions of the channel (see also Figure 21A and B). Part of the lumenal domain was found to be positioned over the central pore of the channel (Figure 25A, middle panel).
In addition, channels derived from native membranes appeared to be elliptical and larger; 125Å in the longest dimension compared to 85Å of the purified Sec61p channel.
When purified and native channels were overlaid (Figure 25A, right panel) it became clear that the region without the lumenal domain seemed to be similar. The most striking differences were visible where the lumenal domain emerged from the channel. This part of the channel wall is extended and the central pore is enlarged (Figure 25A, top row, right). The increased pore size (25x50Å at a 100% threshold) is consistent with results previously described using freeze-fracture electron microscopy (Hanein et al., 1996).

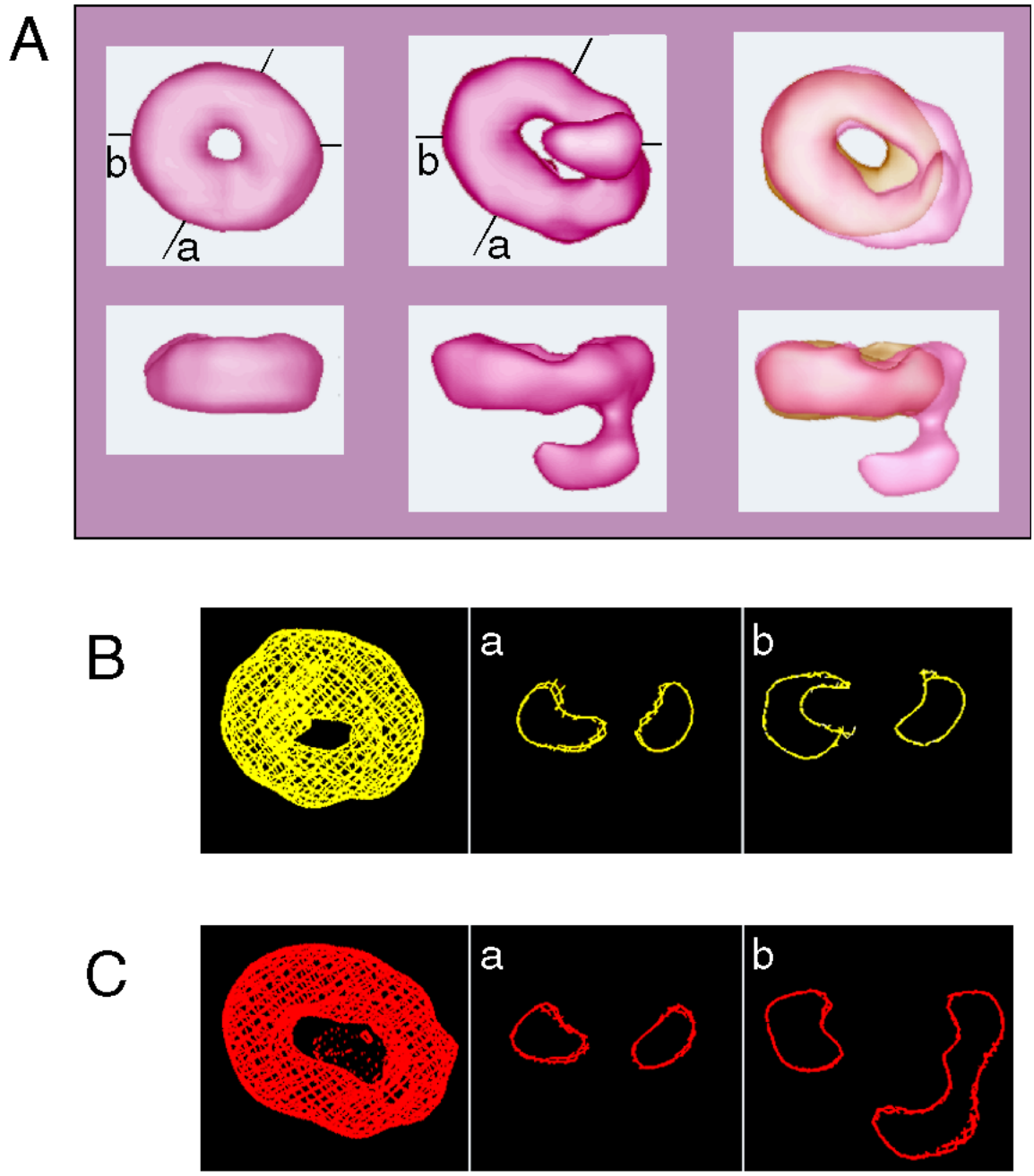

Figure 25: Structure of the translocation channel. (A) 3D maps of purified Sec61p channel lacking a nascent chain (left panel) and of the native channel derived from KRM (middle panel) are shown without bound ribosomes at a threshold level of 110%. The right panel presents an overlay of native and purified channels. In the top row, the channels are shown as viewed from the lumen of the ER, in the bottom row they are shown in frontal view. (a) and (b) indicate the cutting planes of slices through the channels shown in (B) and (C). (B) The purified Sec61p channel lacking a nascent chain is shown as a wiremesh surface viewed from the ribosome (left panel). The middle and right panels present slices through the channel which correspond to views cut along planes equivalent to those indicated in (A). (C) Similar to (B), except that the translocation channel derived from KRM is shown.

To study the actual pore in more detail, we compared purified and native translocation channels after sectioning them along the axes marked (a) and (b) in Figure 25A. The Sec61p channel derived from proteoliposomes containing purified membrane proteins has a cup-shape with a larger opening towards the ribosome (about 50Å diameter) and a more narrow opening (about 25Å) towards the lumen of the ER (Figure 25B, middle and right panel). The channel derived from native membranes looked similar when sectioned along the short axis (Figure 25C, middle panel, a in Figure 25A). However, a section cut 60° away (b in Figure 25A) revealed a much larger opening throughout the channel (Figure 25C, right panel).

3.3.7. The Nature of the Lumenal Domain

One of the most interesting features seen in structures derived from the native ER membranes is the additional lumenal domain. The position of the lumenal domain with respect to the central pore of the translocation channel seems to allow contact with the nascent chain when it is emerging from the channel. Therefore, the lumenal domain might be actively involved in protein translocation. We wished to identify the component of the translocation channel that forms the lumenal domain. Thus, we analyzed the protein composition of ribosome-channel complexes prepared from KRM. To release non-ribosomal proteins associated with the ribosome-channel complexes, we performed an extraction with very high salt concentrations (1200mM potassium acetate) and puromycin (to release the nascent chains). We assumed that all proteins that remain ribosome-associated under the conditions we used for isolating the ribosome-channel complexes (500mM potassium acetate, 1.5% digitonin) would most likely be integral membrane proteins. Thus, to enrich hydrophobic proteins, the sample was extracted with Triton X-114 before separation by SDS-PAGE. Staining with Coomassie Blue revealed that only two other complexes of membrane proteins are present at a concentration similar to the one of the Sec61p complex (Figure 26, lane 2).

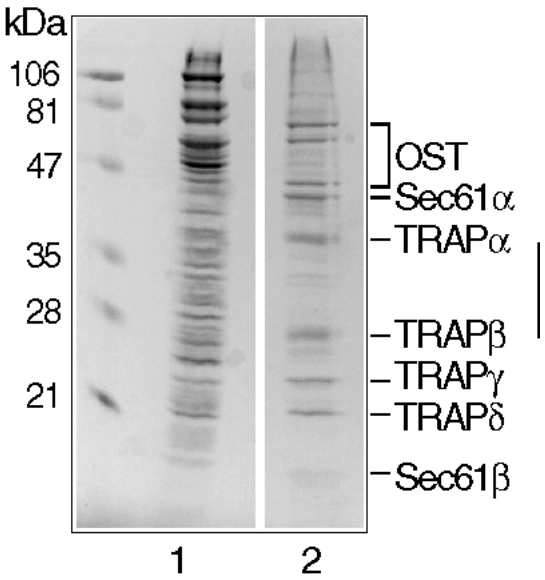

Figure 26: Ribosome-associated membrane proteins present in ribosome-channel complexes derived from KRM. Ribosome-channel complexes derived from KRM were treated with 1200mM potassium acetate and puromycin to release the nascent chains and extract ribosome-associated proteins. After the high salt/ puromycin treatment, the ribosomes were pelleted and the supernatant was extracted with Triton X-114 to enrich hydrophobic proteins. The samples were analyzed by SDS-PAGE and Coomassie staining. For comparison, an aliquot of KRM is shown in lane 1. Lane 2 shows proteins that have been extracted from ribosome-channel complexes by the treatment described above. In addition to the Sec61p complex (Sec61α and β are indicated), three subunits of oligosaccharyl transferase (indicated by OST) have been found and the four subunits of the TRAP complex (marked by TRAPα, β, γ, δ).

One protein complex was identified as oligosaccharyl transferase (OST; Kelleher et al., 1992) which attaches carbohydrate chains to newly translocated nascent chains. Three proteins of the OST were present in the ribosome-channel complexes: P48 and ribophorin I and II. The other membrane proteins were identified as the four subunits of the translocon associated protein complex (TRAP; Wiedmann et al., 1987; Görlich et al., 1990).

4. DISCUSSION

In this thesis project, cotranslational ER targeting of secretory proteins and binding of ribosomes to the ER membrane was studied. First, calmodulin was described as a novel cytosolic interaction partner for signal sequences. Next, ribosome binding to the membrane of the endoplasmic reticulum was studied, resulting in a new view of how differences in binding result in specificity of translocation. Last, a structural analysis of ribosome-translocation channel complexes with and without a nascent chain gave new insights in the nature of the ribosome-membrane junction and the anatomy of the channel.

4.1. Interaction of Calmodulin with Signal Sequences

We have found that the cytosolic protein calmodulin binds to signal sequences of nascent polypeptide chains in a Ca^{2+} dependent manner. The interaction is sensitive to inhibitors of calmodulin function. When SRP or microsomes are present, calmodulin binding to signal sequences is reduced.

4.1.1. Substrate Binding by Calmodulin

Calmodulin is a small cytosolic protein, known to be a key-player in the modulation of Ca^{2+} dependent signal transduction pathways by regulating the activity of many different enzymes, such as calcineurin, calmodulin kinase I and II or elongation factor 2 kinase (for review see James et al., 1995).
Based on the crystal structure of calmodulin (Babu et al., 1985; Babu et al., 1988) and on data derived from photo-labeling studies (Kauer et al., 1986; O'Neil et al., 1989; O'Neil and DeGrado, 1989) it is well understood how calmodulin binds its substrates (reviewed in O'Neil and DeGrado, 1990). Upon Ca^{2+} association with calmodulin, a conformational change creates a peptide binding site by bringing two hydrophobic patches closer together which are located in the globular domains at the N- and C-termini. The extended α-helix connecting the N- and C-terminal lobes could serve as a flexible tether to adjust the peptide binding domain

to a wide range of substrates with a high sequence variability. A common feature of most calmodulin binding motifs is a peptide adopting an amphiphilic α-helical structure (so-called baa-peptides, for basic, amphiphilic α-helices). It has been suggested that signal sequences can adopt α-helical structures (Gierasch, 1989; McKnight et al., 1989; Plath et al., 1998), maybe explaining how calmodulin can recognize signal sequences as a substrate.
Using model peptides carrying a photoaffinity label it has been shown that two methionine residues of the C-terminal lobe and another methionine residue at the N-terminal lobe are contacting hydrophobic residues of bound peptides (O Neil et al., 1989). For SRP, similar to calmodulin, it has been suggested that methionine residues are involved in recognition of substrates (Bernstein et al., 1989). The methionine-rich M-domain of the 54kDa subunit of SRP (SRP54) contains the signal sequence binding site (Zopf et al., 1990; Lütcke et al., 1992). In fact, the crystal structure of Ffh, the prokaryotic homologue of SRP54 has revealed that the M-domain forms a deep hydrophobic groove lined with highly flexible methionine side chains (Keenan et al., 1998. Given that both calmodulin and SRP seem to use the same principle for association with potential substrates, it is not too surprising that nascent signal sequences can interact with calmodulin. However, it is unlikely that SRP will bind to targets of calmodulin since a stable association of SRP with signal sequences requires the presence of a ribosome.

There is also evidence that the hydrophobic patches at N- and C-termini of calmodulin interact simultaneously with opposite ends of the peptide (O'Neil and DeGrado, 1989). Using a site-specific photocrosslinking approach, we have shown that crosslinks to calmodulin from different positions in the signal sequence each have a distinct mobility when separated by SDS-PAGE. These different mobilities frequently result from crosslinks to different domains of the crosslinking partner. In fact, in S. cerevisiae it has been shown that crosslinks of different mobility from the signal sequence of a secretory protein to the α-subunit of the Sec61p complex correspond to crosslinks to either one of two different membrane anchors of Sec61α (Plath et al., 1998). They occur with a periodicity of three to four amino acids, suggesting that the signal sequence is in an α-helical conformation that contacts one domain of Sec61α on one side and another domain of Sec61α on the other side. Strikingly, our

preliminary data indicate that a similar crosslinking pattern can be observed in photocrosslinking experiments using signal sequences bound to either SRP or calmodulin. This could suggest a general mechanism for signal sequence recognition in which at least two regions of the binding partner must contact appropriate regions of the substrate to allow a stable interaction. Experiments using modified calmodulin and SRP with an internal cleavage site for a site-specific protease, for example factor Xa, could allow mapping of domains interacting with signal sequences and would subsequently lead to a more thorough understanding of the mechanism of signal sequence binding and recognition.

4.1.2. Protein Translocation And Calmodulin

Binding of signal sequences by calmodulin has only been observed in *in vitro* experiments. It would be interesting to know whether this interaction can also occur *in vivo* and whether calmodulin is involved in the cotranslational translocation pathway. We have shown that *in vitro* signal sequences are preferentially bound by SRP when both SRP and calmodulin are present, suggesting that SRP has a higher affinity for signal sequences. However, in the cell there is at least a 1000 fold excess of calmodulin over SRP (10-100μM versus 10nM; Martoglio et al., 1997; Siegel and Walter, 1988) indicating that in spite of the lower affinity calmodulin may bind to signal sequences *in vivo*. Our data also show that calmodulin can easily dissociate from signal sequences and that the interaction of calmodulin and signal sequences would not interfere with translocation of nascent chains across the ER membrane.

In S. cerevisiae, *CMD1*, the gene encoding the calmodulin homologue, is essential (Davis et al., 1986). Using a yeast strain with a temperature-sensitive allele of *CMD1*, it has been shown that the biogenesis of the vacuolar protein carboxypeptidase Y and of the secretory protein invertase is not dependent on the function of calmodulin (Kübler et al., 1994). Yet, for invertase we have demonstrated that calmodulin can interact with the nascent polypeptide chain *in vitro*. Assuming that the primary cytosolic interaction partner of signal sequences in mammalian cells is SRP, it is conceivable that binding of signal sequences by calmodulin does not play a role under normal conditions but that it might become important under certain cell stress

conditions. In the event of an increased expression of proteins destined to cross or to be integrated into the membrane of the endoplasmic reticulum, the amount of available SRP might not be sufficient to mediate targeting. In this case, calmodulin might bind to signal sequences.

In the cell, calmodulin is involved in modulation of Ca^{2+} dependent signal transduction pathways, raising the question of whether the interaction of signal sequences with calmodulin could affect any of these pathways. If we assume that calmodulin can function as a backup binding partner for signal sequences in the case of an overload of signal sequence-bearing nascent chains, this interaction could either trigger inhibition of protein translation or stimulation of the expression of proteins involved in targeting or ER translocation.
In fact, for the unfolded protein response in S. cerevisiae it has been shown before that modulation of translation can result from an overload of secretory proteins (for review see Sidrauski et al., 1998).

Supporting our finding that signal sequences are substrates for calmodulin, another study demonstrated that calmodulin can interact with fragments of signal sequences that have been cleaved off nascent chains during translocation across the ER membrane and that subsequently have been released into the cytosol (Martoglio et al., 1997). Again, the *in vivo* function of this interaction remains elusive. Martoglio et al. speculated that fragments of a few specific signal sequences are released into the cytosol to function as calmodulin antagonists. However, based on our results we consider it likely that binding to calmodulin is an intrinsic property of most if not all signal sequences. Clearly, more work needs to be done to understand if and how the interaction of calmodulin with signal sequences is important in secretion of proteins from the cell.

4.2. Regulation of Ribosome Binding to the ER Membrane

We have found that SRP-independent targeting of ribosome-nascent chain complexes to the membrane of the ER occurs in the presence of all cytosolic factors, including the nascent polypeptide-associated complex (NAC). Furthermore, we have

demonstrated that in a system containing complete cytosol but lacking SRP, all ribosomes compete for the same membrane binding sites, independent of whether or not they carry a nascent chain with a signal sequence. In the presence of SRP, RNCs with a signal sequence or a signal anchor have a competitive advantage over other ribosomes. However, binding of SRP to the signal sequence is not sufficient for gaining the advantage; in addition, the nascent chain has to insert productively into the translocation channel.
Experiments using reconstituted proteoliposomes containing purified components of the translocation machinery have shown that the Sec61p complex and the SRP receptor are necessary and sufficient for conferring the competitive advantage to RNCs with bound SRP. We have also found that all ribosomes that can interact only loosely with the Sec61p complex behave identical with respect to binding to the ER membrane. It does not matter whether these are nontranslating ribosomes, RNCs of cytosolic proteins or ribosomes carrying a nascent chain with a signal sequence that is not yet long enough to be recognized by the translocation channel.
Our data also suggest that in the absence of SRP there is little exchange between the pools of free ribosomes and ribosomes bound to the ER membrane. In the presence of SRP, however, RNCs carrying a signal sequence with bound SRP can displace loosely bound ribosomes. In contrast, ribosomes translating cytosolic domains of membrane proteins cannot be competed off by these RNCs.

4.2.1. SRP-Independent Targeting in the Presence of Cytosol

Based on experiments employing salt-washed RNCs, NAC has been suggested as a general cytosolic inhibitor of ribosome binding in the absence of SRP (Wiedmann et al., 1994; Lauring et al., 1995a). Addition of SRP would allow RNCs with a signal sequence to overcome the inhibitory effect of NAC (Lauring et al., 1995b).
Our results do not support a model in which specificity of targeting is achieved by NAC-mediated inhibition of ribosome binding. We rather suggest that SRP functions as a positive effector to allow efficient targeting of RNCs carrying a signal sequence, in spite of non-selective binding of ribosomes to the ER membrane. Although we cannot explain the divergence of the results we believe that experiments in a

complete translation system may be more meaningful than experiments using salt-washed RNCs, as they should more closely reflect the physiological conditions.

We have demonstrated SRP-independent targeting in two translation systems. However, based on early studies, targeting was always believed to be dependent on SRP (Walter and Blobel, 1980; Walter et al., 1981). This paradox can be explained by three major differences in the experimental settings- all three resulting in a higher efficiency of RNC binding to the ER membrane in the absence of SRP.
The first difference is the use of PKRM in our system. PKRM are microsomes that have been stripped of endogenously bound ribosomes and RNCs. They offer a higher concentration of unoccupied ribosome binding sites compared to previously used microsomes covered with either endogenous ribosomes (RM, KRM) or ribosome remnants (EKRM). A second difference might be the enhanced efficiency of current translation systems. An increased ratio of RNCs to total ribosomes would result in an increased number of RNCs bound to the ER membrane even when all ribosomes compete for the same binding sites. The last difference is uncoupling of targeting from translation. In our experiments truncated nascent chains of optimal length for productive membrane interaction were used, as a result creating a large time window for insertion into the membrane. Also, when targeting is initiated after translation has been stopped, a higher concentration of microsomal membranes can be added since their inhibitory effects on translation can be neglected.

Based on these assumptions we have to ask how significant SRP-independent targeting is in the context of a cell. There, translation and translocation would occur simultaneously. Hence, SRP-independent targeting would be reduced to a small time window before elongation of the nascent chain, since folding of the polypeptide would result in a conformation in which the signal sequence is no longer available for a productive interaction with the Sec61p complex. Nevertheless, the fact that we have found no evidence for a cytosolic inhibitor of ribosome-membrane interaction suggests that all ribosomes can bind to the ER membrane *in vivo*. This hypothesis is supported by the observation that a sizable population of ribosomes is removed from rough microsomes under high salt conditions (Kalies et al., 1994). Our data indicate

that RNCs with a signal sequence and bound SRP could simply displace these ribosomes, consequently securing a channel for translocation of the nascent chain.

4.2.2. How Does an SRP-Advantage Work?

Experiments using reconstituted proteoliposomes containing purified membrane proteins showed that the interaction of SRP with its receptor is necessary to allow preferred binding of RNCs to the Sec61p complex. But how does the SRP-SRP receptor interaction confer a competitive advantage to these RNCs?

A simple explanation would be that binding of SRP to SR provides an additional link between the RNC and the membrane. We have shown that mere binding of SRP to RNCs is not sufficient for getting an advantage over competing ribosomes. RNCs of the mutant ppl86Δ13-15 chain do not gain a SRP advantage although photocrosslinking experiments have shown that the mutant signal sequence still binds SRP with an efficiency of 70% compared to wildtype ppl86 (Jungnickel and Rapoport, 1995). Certainly, the affinity of the mutant signal sequence for SRP could be lower than the crosslinking data suggest. However, likewise no SRP advantage is given to RNCs carrying ppl59, nascent chains that interact well with SRP but are too short to insert into the translocation channel (Jungnickel and Rapoport, 1995).

One could speculate that binding of SRP to its membrane receptor grants a kinetic advantage, thus creating a larger time window for a productive interaction of the nascent chain and the Sec61p channel. Once the signal sequence would be recognized by Sec61p and inserted into the channel, the ribosome would remain tightly bound to the membrane. This could explain why in a case where the signal sequence cannot insert properly, such as for ppl59 or ppl86Δ13-15, the kinetic advantage provided by the SRP-SR interaction would not be sufficient to allow successful competition. In contrast, in an *in vivo* situation the time window might be large enough so that RNCs whose nascent chain is initially too short for binding of the signal sequence by the Sec61p complex, may elongate the nascent chain and insert it productively into the translocation channel.

The model described above is based on the assumption that at the ER membrane rapid association and dissociation of ribosomes take place. Both on and off rate of ribosome binding to the Sec61p complex would be high. In contrast, our data show that the exchange between the pools of free ribosomes and those bound to the ER membrane is rather slow. Since ribosome association to the ER membrane is a fast process, slow dissociation is presumably responsible for the low exchange rate. Provided that indeed the off-rate of ribosome binding to the ER membrane is low compared to the on-rate, our data indicate that the SRP advantage might be based on a more complex mechanism. We have shown that RNCs of ppl86 with bound SRP can displace nontranslating and other loosely bound ribosomes from the ER membrane. The fact that in the presence of SRP the displacement occurs so rapidly could indicate that it is an active process. The only membrane components necessary for gaining an SRP-advantage are the SRP receptor and the Sec61p complex. Therefore they might cooperate in a so far unknown manner to actively displace other ribosomes to secure a translocation site for RNCs with a signal sequence and bound SRP. Our data derived from experiments with ppl59 and ppl86Δ13-15 also show that the SRP advantage does not originate from the initial interaction of RNCs with bound SRP and membrane but that it is implemented at a later point. This point may be the assembly or the gating of the translocation channel.

The Sec61p complex is thought to form the translocation channel by assembling into trimeric or tetrameric structures (Hanein et al., 1996) but otherwise little is known about the dynamics of translocon formation. One could envision that to create a new translocon the SRP receptor can recruit subunits from a multimeric channel even if this channel has a ribosome bound to it. The insertion of the nascent chain into the translocation channel would stabilize the newly formed structure. Our data show that ribosomes synthesizing cytosolic domains of membrane proteins cannot be displaced from their translocation sites. This is true even when the physical connection between ribosome and preceding membrane anchor is disrupted by cleavage with a site-specific protease, suggesting that under these conditions the translocon remains stable. If SRP receptor plays an active role in recruiting subunits for a new translocon it will most likely not be able to do so from stabilized channels.

In summary, we suggest that SRP and SRP receptor could actively assist in the recruitment of a translocation site for RNCs with a signal sequence. As has been proposed before, insertion of the signal sequence into the translocation channel would alter the channel and/or the ribosome-channel junction. The new arrangement would not depend anymore on the presence of a nascent chain in the channel and therefore comprise more than just a link between ribosome and channel provided by the nascent chain.
Based on these results, one could have expected significant changes in the ribosome-bound translocation channel after insertion of the nascent chain. However, at a resolution of 25-27Å, a structural analysis of ribosome-channel complexes with and without an inserted nascent chain (see below) did not reveal major differences. Further experiments exploring the function of SRP receptor and the dynamics of the translocon in conjunction with a high resolution 3D structure of the ribosome-channel complex will probably allow a better understanding of membrane binding of RNCs with bound SRP compared to binding of other ribosomes.

4.2.3. Other Aspects of Unspecific Ribosome Binding to the ER

As mentioned before, the fact that we failed to detect an inhibitory effect of cytosolic factors on binding of ribosomes to the membrane of the ER probably means that most translocation sites on the ER surface are occupied at all times. Again, this would not hinder efficient translocation of secretory proteins across the ER membrane since SRP binding allows RNCs with a signal sequence to displace most other ribosomes from the membrane.
Therefore, both nontranslating ribosomes and ribosomes synthesizing a variety of proteins could become associated with the membrane of the endoplasmic reticulum. In fact, translation could start at the ER membrane. It has been shown that even nascent chains ultimately destined for the cytosolic compartment can be translated by ribosomes bound to the ER membrane. These nascent polypeptide chains would most likely not enter the lumen of the ER since the Sec61p complex executes a second signal sequence recognition step (Jungnickel and Rapoport, 1995). Therefore, polypeptides without a functional signal sequence are excluded from translocation across the ER membrane.

Little is known about what is happening to a translocon after translation and subsequently translocation has been terminated. The ribosome may remain bound to the membrane until it is displaced by a RNC with bound SRP. Alternatively, it could dissociate from the translocation channel after termination of translocation. Similarly, the translocon with all associated factors could disassemble with the components returning to a pool of free subunits or it could stay in the assembled state for another round of translocation.
In freeze-fracture images of PKRM, ring-like structures of the Sec61p complex were detected even though in these microsomes the majority of ribosomes had been removed by treatment with puromycin and high salt (Hanein et al., 1996). These data suggest that even after dissociation of the ribosome the translocation channel remains intact, although the release of the nascent chain by puromycin may not reflect physiological termination of translation.

4.2.4. Consequences for Integration of Multispanning Membrane Proteins

So far, two models existed for how the next transmembrane domain is integrated into the membrane after a cytosolic loop has been synthesized. According to the first model, the ribosome leaves the translocation site to translate the cytosolic domain (Blobel, 1980; Sabatini et al., 1982; Kuroiwa et al., 1996). The following membrane anchor is bound by SRP when it emerges from the ribosome. Subsequently, the RNC is retargeted to the same or a different Sec61p channel. However, so far there is no evidence that the integration of multispanning membrane proteins depends on SRP other than for the initial targeting step.
The second model assumes that the ribosome never leaves the translocation site and that the cytosolic domain is synthesized by a membrane bound ribosome (Wessels and Spiess, 1988; Mothes et al., 1997). This model is supported by our finding that ribosomes translating a cytosolic domain of a multispanning membrane protein will not be displaced by any other ribosomes, even if they carry a nascent chain with a signal sequence and bound SRP.

4.3. 3D Structures of Ribosome-Translocation Channel Complexes

We have performed a structural analysis of loose and tight stages of ribosome binding to the membrane of the endoplasmic reticulum using single particle cryo electron microscopy. In this study, 3D structures of ribosome-channel complexes in detergent solution were generated in the presence or absence of nascent chains. The translocation channels were either derived from vesicles containing purified Sec61p complex or from native microsomes.

Several common features were observed in all 3D structures examined. The translocation channel is assembled into a disc containing a central pore and precisely positioned with respect to the ribosome. As a result, the channel pore is aligned with the exit site of the ribosomal nascent chain tunnel, providing a continuous passage for the nascent polypeptide from the peptidyltransferase center into the lumen of the ER. A discrete number of connections link the large ribosomal subunit to the translocation channel, bridging a sizable gap between ribosome and channel.
To our surprise, no significant differences were seen between ribosome-channel complexes carrying or lacking a nascent chain at a resolution of 25-29Å. Instead, we found that channels derived from native microsomes or from vesicles containing purified Sec61p complex differ remarkably.

4.3.1. The Ribosome-Membrane Junction

In all ribosome-channel complexes analyzed, a gap of about 20Å was seen between the ribosome and the translocation channel. This result is somewhat surprising. Based on earlier biochemical studies, a continuous seal had been predicted for the ribosome-membrane junction, particularly in the presence of a membrane-inserted nascent chain when gating of the translocation channel was thought to induce a much more intimate ribosome-channel junction (Crowley et al., 1994). In contrast, we show that even with a nascent chain the connection between ribosome and channel is established by a discrete number of links conserved between mammals and yeast. These links are arranged horseshoe-like around the pore of the channel with the opening of the horseshoe providing a passage to the cytosol.

On the ribosomal side, the links are connected to distinct high density features, most likely representing RNA. This is in good agreement with recently published data, showing that the 28S RNA of the large ribosomal subunit mediates ribosome binding to the membrane of the ER (Prinz et al., 2000). Specific RNA sequences are involved, supporting the idea that a limited number of connections in conserved positions exist. It seems possible that the links between ribosome and translocation channel that are visible in the 3D maps are actually formed by ribosomal RNA rather than proteins.

For several reasons we consider it unlikely that the gap between ribosome and translocation channel is an artifact. First, it has been observed in two independent studies, with different sources of material as well as different methods of complex preparation and analysis (Beckmann et al., 1997 and this thesis). Second, the gap is present in 3D maps calculated either with compensation of the contrast transfer function (Beckmann et al., 1997) or by restricting the resolution, such that the contrast transfer function does not play a critical role (this study). Third, the gap is still seen at unrealistically low threshold levels. Fourth, a statistical analysis of the structures showed that with a confidence greater than 99% the gap is free of any mass. Fifth, the gap was also seen in 3D maps of yeast ribosome-channel complexes in intact, not detergent-solubilized membranes (our unpublished observation), excluding the possibility that it is an artifact introduced by using ribosome-channel complexes in detergent solution. Preliminary data indicate that the gap is also visible in ribosome-channel complexes of intact salt-washed mammalian microsomes, providing further evidence that the ribosome-membrane junction does not change in the presence of a nascent chain.

At the resolution of the 3D maps presented here, the environment in the gap is difficult to evaluate. It is possible that some low density material is present. Weakly ordered and flexible segments of either proteins or RNA could reach into the gap. Also, individual α-helices or amino acid side chains would not be detectable at a resolution of 25-29Å. However, based on the facts presented above, it seems likely that a gap exists through which nascent chains or small molecules could pass.
The existence of a gap does not necessarily contradict previous data suggesting that the ribosome-membrane junction is sealed towards the cytosol. It has been shown

that polypeptides passing into the lumen of the ER are protected against externally added proteinase K. Indeed, the gap would be too narrow to allow fully folded proteinase K to enter it. Likewise, electrophysiological experiments have revealed that in the presence of nascent chains the flow of ions through the membrane is prevented (Simon and Blobel, 1991). Assuming that the block occurs within the membrane, possibly mediated by the nascent chain itself, the gap should not jeopardize the identity of the ER lumen. It is more difficult, however, to bring our data in agreement with data derived from fluorescence quenching experiments that suggest the existence of a seal for ions. For example, fluorescent probes in short preprolactin chains of 56 or 64 amino acids on membrane-bound ribosomes could not be quenched by iodide ions added to the cytosolic compartment (Crowley et al., 1994). Yet, these data are in apparent contradiction with the fact that these chains can be trimmed to about 30 residues by externally added proteinase K (Jungnickel and Rapoport, 1995). So far, we cannot explain the discrepancy. It seems worth considering that based on the experimental design it might be much easier to detect the passage of an extended nascent chain through the gap than the passage of iodide ions, which are supposed to collisionally quench a fluorescent dye.

The efficiency of collisional quenching depends on the concentration of the quenching agent and its free diffusion (Hamman et al., 1997). For successful collisional quenching of fluorescent groups in nascent chains, most likely free diffusion of iodide ions is needed in all 3 dimensions around the fluorescent probe. In addition, the concentration of iodide ions in the immediate vicinity of the probe must approach the concentration in the bulk solution. Assuming that hydrated iodide ions have a diameter of about 9Å (Hamman et al., 1998) it is difficult to envision how these requirements can be fulfilled in a gap that is about 20Å wide. Furthermore, the negatively charged iodide ions would probably experience some repulsion if ribosomal RNA is indeed involved in forming the ribosome-membrane junction.

In contrast, for a nascent polypeptide the constrains for entering the gap might be less critical. A hydrated extended polypeptide chain presumably has a diameter of 10-12Å (Hamman et al., 1997) suggesting that there might be some steric barriers to entry. However, the nascent chain might extend toward the cytosol if with ongoing elongation the space between the ribosomal tunnel exit and the translocon becomes insufficient to accommodate the polypeptide. Certainly this could be the case for a

nascent chain with a signal sequence that is too short to insert properly into the translocation channel. Since this nascent chain cannot induce gating of the channel, the resistance to enter the plane of the membrane might be greater than the steric hindrance to enter the gap and subsequently the cytosol. Upon further elongation the nascent chain would reprobe the translocon, the signal sequence could insert into the membrane and would be recognized by Sec61p and as a result open the channel for translocation of the polypeptide into the lumen of the ER.

Once the channel is open, it is probably more favorable for the nascent chain to enter it instead of looping out into the gap. This could also explain why the energy provided by protein translation is sufficient to allow directional translocation in the presence of a gap between ribosome and channel.

When a cytosolic domain of an integral membrane protein is synthesized, its translocation across the membrane is hindered because the membrane anchor functions as a stop-transfer sequence. In this case, the gap would permit the exit of the polypeptide into the cytosol without removal of the ribosome from the translocation site. A similar model would apply to translocational pausing during synthesis of certain secretory proteins, such as apolipoprotein B (Hegde and Lingappa, 1996). Here, a non-hydrophobic “pause-transfer” sequence stops polypeptide movement through the channel and for a short period the newly translated polypeptide chain gains access to the cytosol, presumably through the gap in the ribosome-membrane junction.

The gap between ribosome and channel could also be used by membrane-bound ribosomes translating cytosolic proteins. Since resistance against transfer of these nascent chains across the membrane is very high, the newly translated cytosolic proteins would probably exit the ribosome-membrane junction through the gap.

4.3.2. Shape and Size of the Translocation Channel

Surprisingly, in both ribosome-channel complexes derived from native microsomes or from vesicles containing purified Sec61p complex we did not detect any significant changes in the pore size of the translocation channel in the presence or absence of a nascent chain. Our biochemical data show that the nascent chains are stably bound to the ribosome-channel complexes. However, no nascent chain or ribosome-associated tRNA was consistently detected in the 3D structures. This is not surprising since at a resolution of 25-29Å a single polypeptide chain, that may even be flexible, would not add significant electron-dense mass. Detection of a ribosome-bound tRNA may be equally difficult in a structure derived from single particle analysis since the tRNAs in the sample are presumably found at 3 different sites (Agrawal et al., 1999).

Based on previous biochemical studies it has been suggested that the translocation channel can exist in two different conformations; as a large pore with an estimated size of 40-60Å (Hamman et al., 1997) and as a smaller, presumably inactive pore with a size of about 9-15Å (Hamman et al., 1998).
The largest pore observed in our study was found in channels derived from native membranes and had a size of about 25x50Å. It may correspond to the large pore described earlier after insertion of the nascent chain. However, we have not observed the smaller pore in channels derived from native microsomes, even in the absence of a nascent chain. A possible explanation is that the structure derived from PKRM without a nascent chain may not correspond to the true closed state of the translocation channel, maybe due to detergent solubilization of the membrane during sample preparation.
It is conceivable that the structures derived from purified Sec61p may represent the ground state, since there the channels have a rather small pore of 15-25Å. However, the size of these channels did not change in the presence of a nascent chain, maybe suggesting that the protein that is forming the additional lumenal domain in structures derived from native membranes is necessary to fully open the translocation channel. This additional protein may enhance the efficiency of translocation, although it has been shown that translocation can be reproduced with purified Sec61p complexes in reconstituted proteoliposomes.

Another interesting difference between native channels and those derived from purified Sec61p is the shape of the cross-section. Purified Sec61p channels show a cup-like cross-section with a wider opening towards the ribosome and a somewhat constricted opening towards the ER lumen. A very similar shape has been described for the Sec61p channel purified from S. cerevisiae (Beckmann et al., 1997). The narrow lumenal opening might minimize the transfer of small molecules through the membrane, whereas the wider opening could collect the growing nascent chain and allow the signal sequence to be in an optimal position for presentation to the Sec61p complex.
In contrast to the channel derived from purified Sec61p, the native channel shows a much wider pore that may be more suitable for ongoing translocation. Again, recruitment of the additional protein into the channel might be necessary to convert it from one state into the other.
Identification of this additional protein and reconstitution experiments with the purified factor might help to clarify its role in the assembly of the larger translocation channel.

4.3.3. A Lumenal Domain Associated with the Native Sec61p Channel

In all 3D structures derived from native microsomes, a prominent lumenal domain was present. It appeared to be precisely oriented with respect to both ribosome and channel. The tip of the lumenal domain was positioned under the channel pore, as to contact the nascent chain upon its entry into the ER lumen. We consider it unlikely that this domain is formed by a soluble protein resident in the ER lumen, since it remains bound to the ribosome-channel complex even under high salt conditions in the presence of detergent. In addition, as suggested by a comparison of native and purified channel, the lumenal domain may be part of an integral membrane protein that is intercalated into the Sec61p ring.
So far, the identity of the protein is unknown. However, we have identified candidate proteins. Only two membrane protein complexes other than the Sec61p complex are found in the native ribosome-channel complexes used for single particle analysis. The first candidate is the oligosaccharyl transferase complex (OST; Kelleher et al., 1992). Of its components we have found ribophorin I and II and the smaller protein P48 in

the native ribosome-channel complexes. Since OST transfers carbohydrate chains onto consensus sites of the nascent chains immediately after its transfer through the membrane, it is most likely found in close vicinity of the translocation channel. Nevertheless, ribophorin I and II together with P48 are very likely too large to form the lumenal domain. As a second complex of membrane proteins we identified the so-called TRAP complex (for translocon-associated protein; Wiedmann et al., 1987). All four subunits of the TRAP complex have been shown to be present in ribosome-channel complexes derived from native microsomes. Both TRAPα and TRAPβ contain large lumenal domains (Görlich et al., 1990). This together with the overall size of the complex makes TRAP a good candidate for the additional protein present in translocation channels derived from native membranes. So far, the function of the TRAP complex is unknown. However, using a photocrosslinking approach it has been shown that TRAPα is located in close proximity to the nascent chain during its transport across the membrane (Wiedmann et al., 1987; Krieg et al., 1989; Wiedmann et al., 1989). Although the TRAP complex is not required for translocation of the secretory protein preprolactin (Görlich and Rapoport, 1993) and is not essential for translocation of all substrates tested so far (Migliaccio et al., 1992; Görlich and Rapoport, 1993), it might enhance the performance of the translocation channel. Since both the TRAP complex and the OST complex have been isolated, experiments using proteoliposomes containing purified membrane proteins might be helpful in identifying the partner of Sec61p in translocation of proteins across the ER membrane.

The data presented in this thesis project have shed initial light on the mechanism of ribosome binding to the membrane of the endoplasmic reticulum. We have shown that SRP-mediated targeting comprises more than just guiding the signal sequence-bearing RNCs to the ER membrane. We have also presented a first structural analysis of the ribosome-translocation channel complex in the presence of a translocating nascent chain. Based on our data, some exciting questions are raised. How does a ribosome actively displace another ribosome from a membrane binding site? What is the role of SRP receptor in translocation channel assembly? Which other proteins are

part of the completely assembled channel and what are the dynamics of translocon assembly and disassembly? How can a translocon “remember” to remain available until the integration of multi-spanning membrane proteins is completed?
Some of these questions will be answered when a high resolution map of ribosome-translocation channel complexes is available. A 3D map of ribosomes bound to intact, not detergent solubilized membranes will also provide more information. Clearly, a more detailed knowledge of communication between individual components of the translocon will be needed to better understand this complex system.

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6. APPENDIX

ABBREVIATIONS

CAM	calmidazolium chloride
CTABr	cetyltrimethylammonium bromide
CTF	contrast transfer function
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGTA	ethylene glycol-bis (β amino ethyl ether)
ER	endoplasmic reticulum
eq	equivalent (see Walter and Blobel, 1983b)
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
kDa	kilodalton
KRM	salt washed RM
lep	leader peptidase
mRNA	messenger-RNA
NAC	nascent polypeptide-associated complex
NEM	*N*-ethylmaleimide
PKRM	puromycin/high salt treated RM
PMSF	phenylmethylsulfonyl fluoride
ppαF	preproalphafactor
ppl	preprolactin
RM	rough microsomes
RNA	ribonucleic acid
RNC	ribosome-nascent chain complex
rpm	revolutions per minute
SDS	sodium dodecylsulfate
SDS-PAGE	SDS ployacrylamide gel electrophoresis
SRP	signal recognition particle
SRP54	54kDa subunit of SRP
SR	SRP receptor
TCA	trichloroacidic acid
TFP	trifluoperazine
TRAM	translocating chain associating membrane protein
TRAP	translocon associated protein
tRNA	transfer RNA
UV	ultraviolet light
3D	three dimensional

ERKLÄRUNG

Ich erkläre, daß ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Andrea Neuhof Boston, den 1.Mai 2000

LEBENSLAUF

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1990	Erwerb der allgemeinen Hochschulreife (Abitur) an der Erweiterten Oberschule"G. Dimitroff" in Nauen
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VERÖFFENTLICHUNGEN

Neuhof, A., Rolls, M.M., Jungnickel, B., Kalies, K.U., Rapoport, T.A. (1998) Binding of signal recognition particle gives ribosome/nascent chain complexes a competitve advantage in endoplasmic reticulum membrane interaction. Mol. Biol. Cell 9:103-115.

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EIGENANTEIL AN DER VORGELEGTEN ARBEIT

Teil 1: Calmodulin interacts with signal sequences
und
Teil 2: Regulation of ribosome-binding to the ER membrane

Alle gezeigten Experimente wurden von mir durchgeführt.

Teil 3: Structural analysis of ribosome binding to the ER membrane

Dieses Projekt wurde in Zusammenarbeit mit dem Labor von Christopher W. Akey am Boston University Medical Center durchgeführt.
Ich hatte folgenden Anteil an dem Projekt:
-Reinigung des Sec61p-Komplexes und des SRP-Rezeptors aus rauhen Mikrosomen
-Etablierung und Optimierung der Methodik zur Isolierung von Komplexen aus Ribosomen/naszierenden Ketten/Translokationskanal, die erstmals die elektronenmikroskopische Analyse von translozierenden Ribosomen/Sec61p-Komplexen ermöglichte.
-Präparation aller Proben für die Elektronenmikroskopie.
-Biochemische Analyse der isolierten Ribosomen/Sec61p-Komplexe.
-Die Elektronenmikroskopie und Ermittlung der dreidimensionalen Struktur des Ribosomen/Sec61p-Komplexes wurden von Dr. Jean-François Ménétret, Dr. David G. Morgan and Dr. Chris W. Akey durchgeführt.